Hematology

(REVISED 1962)

U. S. NAVAL MEDICAL SCHOOL

NATIONAL NAVAL MEDICAL CENTER

BETHESDA, MARYLAND

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ELEMENTS CONTAINED IN NORMAL BLOOD

PLASMA	Serum	Antibodies	Other organic and inorganic compounds plus sodium, calcium, potassium, iron, magnesium, chlorides, carbonates, phosphates, sulphates
		Serum albumin	
		Serum globulin	
		Glucose	
		Urea	
	Fibrinogen--Yields Fibrin		
CELLULAR ELEMENTS	Erythrocytes	Hemoglobin	
		Lecithin	
		Salts	
	Leukocytes	Granulocytes	
		Lymphocytes	
		Monocytes	
	Thrombocytes		

ORIGIN OF BLOOD CELLS

During the first month of intrauterine life the blood cells are formed outside the embryo in the blood islands of the yolk sac. These cells are large nucleated hemoglobiniferous cells resembling the rubriblast of the pernicious anemia type. As development continues, these primitive red cells are replaced by smaller cells of the erythrocytic series, which by the second month are being formed from the mesenchyme of the liver. At this time granular leukocytes and megakaryocytes can also be found in the liver. The granulocytes become quite numerous by the fifth month of fetal life. During the fourth month red blood cells are actively formed in the spleen, but erythropoiesis diminishes there during the fifth month. The spleen appears to be concerned with lymphopoiesis from the fourth month and monocytes appear during the fifth month. At about the fifth month granulocyte and thrombocyte formation occurs. In the adult, erythrocytes, granulocytes and thrombocytes originate in the bone marrow, lymphocytes in lymph nodes and spleen, and monocytes probably from the reticuloendothelial tissue, especially of lymph nodes and spleen. Plasma cells are believed to develop by metamorphosis from lymphocytes.

The marrow of practically all the bones is red during the first few years of life, but between the ages of 5 and 7 years the long bones begin to form fatty marrow. By 18 years of age red marrow is confined to the vertebrae, ribs, sternum, skull, pelvic bones and to some extent, the proximal epiphyses of the humerus

and femur. To meet unusual demands the fatty marrow can rapidly become replaced by hematopoietic marrow.

The processes of blood manufacture and blood destruction are going on continuously in the normal individual and the blood is kept in a state of more or less constant cellular composition. Red blood cells survive from 100 to 128 days, granulocytes and thrombocytes can survive to 4 days. The lymphocytes' life span is still subject to study; some apparently live only hours, others days.

The stimulus for red blood cell release into the circulation is probably anoxemia and for leukopoiesis probably some chemotactic factors liberated at the site of tissue injury or destruction of leukocytes.

Certain disease processes result in the production of a large number of a particular type of cell, and this disease stimulus causes the blood forming tissues to pour forth immature cells into the blood stream. The presence of immature cells in the circulating blood contributes to the diagnosis of many diseases. For this reason, a knowledge of the morphology and staining characteristics of the mature and immature blood cells is necessary.

FUNCTIONS OF THE BLOOD

The functions of the blood are so intimately associated with the processes of life that any discussion of the subject is necessarily extremely complex. For practical purposes the function of the blood can briefly be discussed under two main classes.

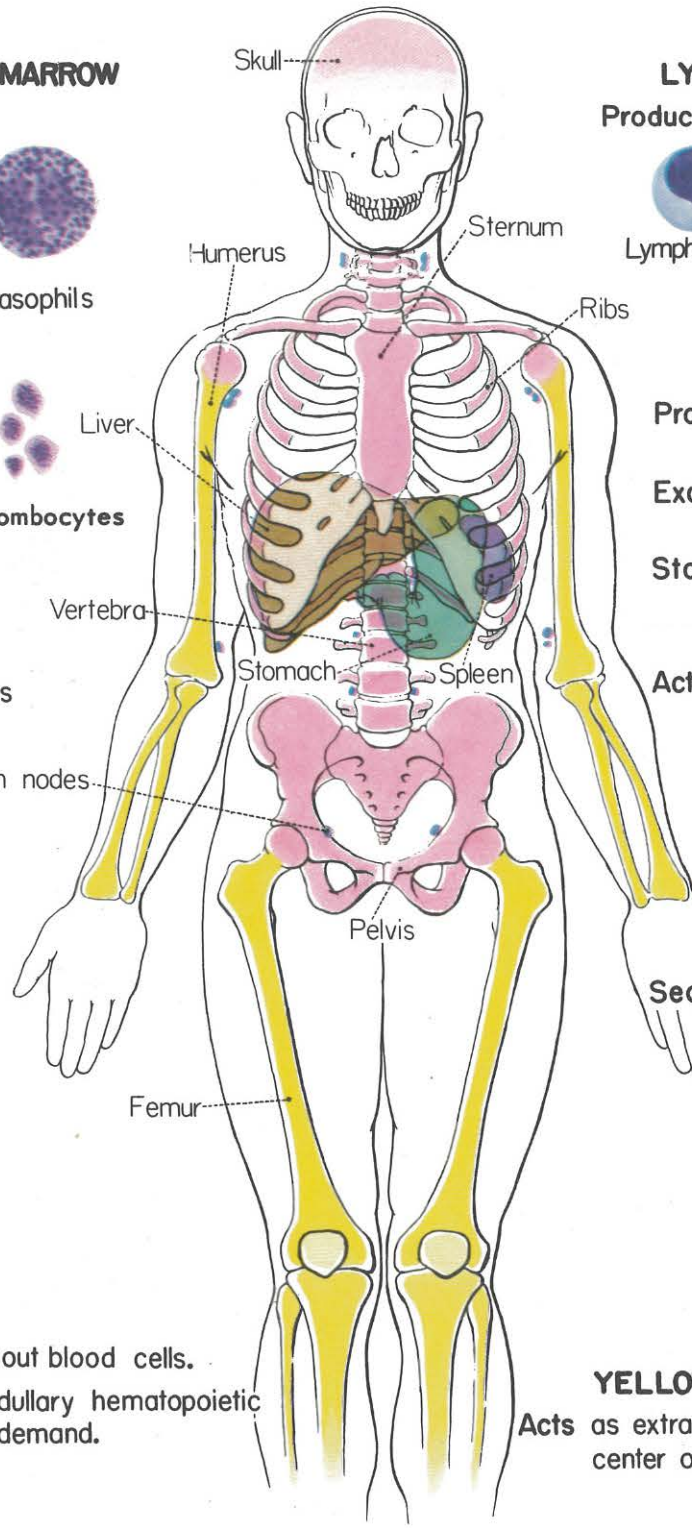
Metabolic Functions

Metabolic functions are the absorption and transportation of food material, water, salts, and oxygen to the tissues; the absorption and transportation of the products of cell metabolism (carbon dioxide, lactic acid, and other waste materials) to the organs of excretion (lungs, liver, skin, kidneys, and intestines); the maintenance of the acid-base equilibrium of the body by the action of buffer salts in solution; the distribution of secretions (insulin, pituitrin, and thyroxin) from the various endocrine glands.

Defensive Functions

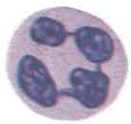
Defensive functions are: (1) the mobilization of the defense mechanisms of the body against invasion by disease processes, as the production and distribution of immune bodies, antitoxins, and phagocytes, and (2) the promotion of healing processes where disease has injured the tissues.

THE HEMATOPOIETIC SYSTEM



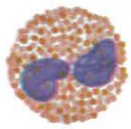
RED BONE MARROW

Produces:



Neutrophils

Basophils



Eosinophils

Thrombocytes

Erythrocytes

LYMPH NODES

Produce:

Lymphocytes

Monocytes

LIVER

Produces:
Prothrombin, fibrinogen

Excretes:
Bilirubin

Stores:
Erythrocyte maturing factor.

Acts as extramedullary hematopoietic center on demand.

SPLEEN

Produces:

Monocytes

Lymphocytes

Destroys: Worn out blood cells.

Acts as extramedullary hematopoietic center on demand.

STOMACH

Secretes:
Intrinsic erythrocytic maturing factor.

YELLOW BONE MARROW

Acts as extramedullary hematopoietic center on demand.

U. S. Naval Medical School '45

ROUTINE HEMATOLOGICAL PROCEDURES

Routine hematology consists of:

1. Hemoglobin determination.
2. White blood cell count and differential.
3. Hematocrit determination.

A red blood cell count is done at the U. S. Naval Medical School only under the special conditions specified below:

1. Infants.
2. Suspected or known cases of blood dyscrasia.
3. Complete hemogram.

COLLECTION OF BLOOD

Draw 7 ml. of venous blood and place 5 ml. in an oxalated tube. Mix the oxalated specimen well. Utilize the blood remaining in the syringe to make differential smears.

Note: Differential smears should not be made from oxalated blood because they show clover-leaf nuclei of lymphocytes and may produce vacuoles in the neutrophils and monocytes.

The above routine must be modified at times when an intravenous withdrawal could cause great inconvenience. Technically, the intravenous withdrawal is a more accurate procedure than any capillary method.

Anticoagulants

Oxalate solution

Ammonium oxalate	1.2 gms.
Potassium oxalate	0.8 gms.
Formalin, 40%	1.0 ml.
Distilled water, q. s.	100.0 ml.

Use 0.5 ml. of oxalate solution for each 5 ml. of whole blood. Place the desired amount in test tube and allow to evaporate. (The purpose of oxalate is to precipitate the ionizable calcium so that prothrombin is not converted to thrombin.)

CLINICAL HEMOGLOBINOMETRY

Hemoglobin is composed of protoporphyrin, ferrous iron (Fe^{++}), and globin. It is normally present in red cells and abnormally in the plasma. The normal range in male is 15 ± 1 gms. per 100 cc.; in females, 14 ± 2 gms. per 100 cc. The average normal is 15.4 gms. per 100 cc. Some methods of measuring hemoglobin are:

1. Cyanmethemoglobin method
2. Oxyhemoglobin method
3. Direct matching method
4. Acid hematin method
5. Copper sulfate falling drop method

At the U.S. Naval Medical School the cyanmethemoglobin method is preferred; this procedure is outlined below. The Haden-Hausser and Sahli methods are also given because of their widespread use.

Cyanmethemoglobin Method

There are two necessary steps in setting up the cyanmethemoglobin method:

1. Calibration of pipets
2. Preparation of a calibration curve for cyanmethemoglobin.

(1) Calibration of Pipets

Equipment

1. Analytical balance
2. Tuberculin or 2-ml. syringe
3. One-hole rubber stopper, size 0 (obtain commercially)
4. Stopcock grease
5. Beaker, 50-ml.
6. Mercury
7. Retort stand and clamp
8. Thermometer
9. Weighing bottle

Procedure

Heavily lubricate the barrel of the syringe with stopcock grease. Tightly fit the tip of the syringe into the broad end of the rubber stopper. Clamp the stopper to a heavy retort stand. Insert the base of the pipet into the other end of the rubber stopper until it is in contact with the tip of the syringe. Adjust the assembly so that the pipet occupies a vertical position.

Allow the mercury and the pipets to reach room temperature before starting the calibration. Determine the temperature of the mercury at the time of calibration.

Pipetting

1. Determine the temperature of the mercury.
2. Slightly withdraw the plunger of the syringe.
3. Hold the beaker containing mercury so that the tip of the pipet is immersed.
4. Withdraw plunger of syringe until mercury reaches 20 cu. mm. mark.
5. Hold the barrel of the syringe steady and quickly pull the beaker of mercury away from the top of the pipet. (Slight fluctuation in the column of mercury at this point has no appreciable significance providing there is no loss of mercury from the tip of the pipet.)
6. Expel the mercury from the pipet into a weighing bottle by manipulation of the syringe. (The weight of the weighing bottle is precisely determined before starting the calibration.)

Weighing

The following procedure must be precisely followed in using the analytical balance.

a. General - Never attempt to use any analytical balance if you are not fully indoctrinated in the use of that particular type of balance.

1. Sit directly before the center of the balance.
2. See that the balance is level.
3. Release and arrest the beam with a slow, steady movement of the hand.
4. Always arrest the beam before placing anything on, or removing anything from, the pans.
5. Find the zero point before each weighing.
6. Do not touch objects to be weighed with fingers. Use tongs.
7. Place the object to be weighed on the left pan.
8. Always close the balance case between addition of weights.
9. When the weighing is finished, raise the beam of the balance and close the balance case.
10. Never move the balance.

b. Technic - For Chainomatic balance.

1. When selecting weight, the first weight is a guess, after that, if by adding the next weight the indicator swings to the right the weight is too light, but if to the left, too heavy.
2. Add weights first by using the dials in the lower right hand corner of the balance. These dials will not turn unless the beam is raised.
3. Then add the lesser weights by means of the chainomatic control. This can be done with the beam lowered.
4. Adjust the chainomatic control until the zero point is reached.
5. The sum of the weights indicated on the upper and lower dials constitutes the final weight of the object.

Calculations

1. Determine the weight of the pipeted mercury by subtraction: (weight of weighing bottle + mercury) - (weight of weighing bottle).
2. Convert grams to milligrams: example: a 20 μl pipet delivered 0.268 gm. of mercury; temp. 20^{o} C.

   $$0.268 \text{ gms. Hg.} \times \frac{1000 \text{ mg.}}{1 \text{ gm.}} = 268 \text{ mg. Hg.}$$

   The terms microliter (μl.), lambda (λ), and cubic millimeter (mm^3) are interchangeable. Microliter is becoming the more acceptable term.

   $$1 \text{ ml.} = \text{cm.}^3$$

   $$1 \text{ ml.} = 1000 \ \mu \text{l.}$$

   $$1 \text{ cm.}^3 = 1000 \text{ mm.}^3$$

   $$1000 \text{ l.} = 1000 \text{ mm.}^3$$

   $$1 \text{ l.} = 1 \text{mm.}^3$$

3. Select the temperature correction factor from Table 1; i.e., the weight of one microliter of mercury in milligrams.
4. Calculate the volume of the mercury, and thus of the pipet, by the following calculation:

   $$\text{Wt. of mercury in mgm.} \times \frac{1 \text{ microliter Hg.}}{\text{No. mg. at } C^{o}} = \text{vol. Hg. in microliters.}$$

TABLE 1

APPARENT WEIGHTS OF MERCURY AND WATER IN AIR

Temperature (degrees Centigrade)	Mercury (weight of 1 μl.)* mgm.
20.0	13.55
21.0	13.55
22.0	13.54
23.0	13.54
24.0	13.54
25.0	13.54
26.0	13.53
27.0	13.53
28.0	13.53

*These values do not indicate the true densities and weights of water and mercury but include corrections for various factors, such as the coefficient of expansion of glass and the buoyant effect of air on the fluid weighed.

5. (Wt. of delivered Hg.) 268 mg. Hg. x $\frac{1\,\mu l.\ Hg.}{13.55\ mg.\ Hg.}$ = 19.8 μl. vol.

   The expected weight for 20 cubic microliters of mercury is 271 mg. at 20°C.

Pipet Accuracy.

1. A 3% error in pipet volume causes an inaccuracy of 0.5 gm. / 100 ml. of hemoglobin.

2. Duplicate pipet calibrations should not vary by more than ± 0.20 μl.

3. It is recommended that pipets with a limit of error of ± 1% be used; discard all others.

CYANMETHEMOGLOBINOMETRY

Cyanmethemoglobin is a stable and accurate standard for clinical hemoglobinometry. The possession of an accurate standard is only one of several essentials for accurate hemoglobin measurement. Of equal importance are the instruments and glassware, the training and attitudes of the operators who perform the measurements, and the proper application of the cyanmethemoglobin standards.

Briefly, the cyanmethemoglobin method consists of the dilution of an accurately measured volume of blood in an accurately measured volume of a solution that will convert hemoglobin to cyanmethemoglobin. The optical density (D) is taken to be directly proportional to the concentration of the pigment.

Each factor involved is considered:

1. Operator

The operator who performs hemoglobinometry should understand the clinical significance of the test and the necessity for a dependable method. He should be well trained in the performance of critical volumetry. He should be familiar with the performance of his instrument so that he is able to identify its misbehavior.

2. Cuvettes

All cuvettes used for hemoglobinometry, even those with manufacturer's marks or specifications, should be matched.

a. Round Tubes

(1) Clean and dry several dozen cuvettes and make sure they are not flawed, scratched or etched. Fill each with the same clean, laked blood solution (approximately 0.2 ml. of whole blood in 50 ml. of water).
(2) Set the photometer at the 540 mμ band or insert the appropriate green filter. Place one filled cuvette in the well of the instrument and set the galvanometer beam near the middle of the scale. Rotate the tube, noting the slight movement of the beam. At the midpoint) of the beam's swing, stop and mark the wall of the cuvette (diamond point) with reference to some mark on the housing of the well.
(3) Each of the other cuvettes is now placed in the well and rotated until the reading of the galvanometer beam corresponds with the reading at which the first tube was marked. At this point each succeeding tube is marked in the same way.

(4) Precautions should be taken to prevent scratching the cuvettes when using them or cleaning them. Test tube racks of wood or coated wire are recommended.

b. Square Cuvettes

(1) Prepare the cuvettes by thorough cleansing and inspection for flaws.

(2) Set the photometer at the 540 mμ band. Place one filled cuvette in the well of the instrument and set the galvanometer beam or meter needle near the middle of the scale. Note the reading. Replace the cuvette with another and so on until all cuvettes have been tested and the readings recorded. If inexpensive cuvettes are being used, it should be possible to select from a large lot those with identical readings and reserve them for hemoglobinometry. If expensive cuvettes are being used, it will be necessary to determine the correction factor for each cell and apply this factor in all subsequent determinations and calculations.

For convenience and to insure adequate mixing during hemoglobinometry, the blood and diluent should be mixed in a separate, clean, dry tube and transferred to the cuvette at the time that it is placed in the instrument.

3. Pipets

a. The 0.02 ml. pipet (Sahli) which is used to measure the blood should be accurate to ± 1 per cent. Several supply houses offer 0.02 ml. pipets with a claimed accuracy of this order. However, it would appear advisable to calibrate a few such pipets in order to verify the degree of accuracy and it is mandatory for calibrated pipets to be used when establishing a calibration curve. The pipets should be acid cleaned and thoroughly washed with water at least once a week. They should be washed and thoroughly dried between each measurement.

b. The transfer pipets used to measure the diluent solution should be of a good order of accuracy and preferably within ± 0.5 per cent. Some of the commercially available pipets are well within these limits. (The Bureau of Standards tolerance on 5 ml. pipets is ± 0.2 per cent.) If a burette or automatic pipet is used for this purpose it should be of the same order of accuracy. The brown glass machlet automatic burette has proven satisfactory.

4. Instrument

The instrument should be carefully calibrated for photometry according to the instructions provided by the manufacturer. A simplified discussion of calibration of photometric instruments can be found in most laboratory manuals and textbooks of clinical pathology. For a more detailed discussion of methods of calibration see Drabkin, D. L., Photometry and Spectrophotometry, in Glasser, O. (ed) Medical Physics, 2:1039-1089, Year Book Pub., Chicago (1950).

5. Standards

The cyanmethemoglobin standard solution may vary, so follow instructions provided with them. One standard, for example, contains the equivalent of approximately 60 mg. of hemoglobin per 100 ml. The exact concentration should be on the label. It varies slightly from one lot to another. The spectrophotometric characteristics of cyanmethemoglobin are such that D is directly proportional to the concentration. Therefore, it is theoretically possible to standardize an instrument using only one concentration of cyanmethemoglobin. Practically, it is better to use two concentrations or more, so that a graph can be constructed that is based on several measurements. In order to do this it is necessary to dilute a portion of the standard solution. The following procedure is recommended.

a. For instruments using a 1:251 dilution (20 cu. mm. of blood in 5 ml. of diluent solution). The standard cyanmethemoglobin solution containing the equivalent of 60 mg. of hemoglobin per 100 ml. corresponds to a 1:251 dilution of blood that contains 15 gm. per cent of hemoglobin. A second solution containing the equivalent of 30 mg. of hemoglobin is prepared. Clean and dry two matched cuvettes. Clean and dry a 5 ml. transfer pipet. In the first tube place 5 ml. of the standard measured exactly to the mark on the pipet. Allow the pipet to drain, but do not blow out. Rinse the pipet several times with diluent solution (cf: below) and then fill it exactly to the mark and allow it to drain into the first cuvette. Into the second cuvette pour 5 or 6 ml. of undiluted standard. The measurement need not be accurate. Tightly stopper and seal both tubes. If rubber stoppers are used, they should be clean and free of blood.
b. For instruments using 1:501 dilution (20 cu. mm. of blood in 10 ml. of diluent solution). The standard cyanmethemoglobin solution corresponds to a 1:501 dilution of blood that contains 30 gm. per cent of hemoglobin. This is beyond the range of clinical usefulness. Two dilutions are prepared, one containing 30 mg. per 100 ml. and the other 15 mg. of the standard. Clean and dry two matched cuvettes. Clean and dry a 10 ml. transfer pipet. In the first tube place 10 ml. of the standard solution measured exactly to the mark on the pipet. Allow the pipet to drain, but do not blow out. Rinse the pipet several times

with diluent solution, Carefully measure into each of the two cuvettes 10 ml. of diluent solution. Do not blow out the pipet, but after these measurements are completed drain the last drop from the tip into some clean gauze. With this same pipet mix thoroughly the contents of the first cuvette and transfer exactly 10 ml. to the second cuvette. Tightly stopper or seal both tubes. If rubber stoppers are used they should be clean and free of blood.

c. The concentration of hemoglobin represented by these standard solutions may be calculated from the number printed on the label of the vial. Where the dilution factor is 251, the number on the label should be multiplied by 251 to learn the concentration of hemoglobin in whole blood to which the standard corresponds. The value for the diluted standard would be half of this. Where the dilution factor is 1:501 the same calculation is made, multiplying the number on the label by 501, then dividing by two to get the value for the more concentrated of the two diluted standards, and dividing by four for the other.

When not in use, place the standard cuvettes in a box to prevent scratching or breaking and refrigerate (without freezing). Before use, remove from the refrigerator, warm to room temperature and wipe the surface free of moisture, lint, and finger prints. Square cuvettes and others that cannot be sealed may necessitate transfer of the standard every time the instrument is checked. Because of the small volume of the standard, the cuvettes must be clean and dry and free of rinse water.

Ideally, the instrument should be checked by inserting the standard before and after each day's run. Minor, unimportant variations occur in most instruments, and most operators, after prolonged experience, become familiar with the degree of constancy of their instruments. It is recommended that a weekly schedule of checking with the standard should be instituted.

The standard solution, as issued, is free of bacterial contamination. The solution itself is bacteriostatic though not bactericidal and should remain free of contamination if it is carefully handled. If the solution becomes turbid it obviously is not satisfactory for photometry, and a new standard should be obtained.

Standards of cyanmethemoglobin are commercially available from multiple sources, as well as from the Walter Reed Army Medical Center, Washington 12, D.C.

6. Blood Sample

Blood may be taken from a freely bleeding capillary puncture or from a venous sample. The latter must be thoroughly mixed by gently tipping the tube end over end 10 to 20 times before blood is taken from it.

7. Diluent Solution

The diluent solution used in the cyanmethemoglobin method is made from reagent grade chemicals.

$NaHCO_3$	1.0 gm.
KCN	50 mg.
$K_3Fe(CN)_6$	200 mg.
Distilled water ..... to	1 liter

The solution should be relatively fresh. Do not prepare more than one month's supply at a time. Keep the solution in a brown bottle. The solution is clear and pale yellow in color. If it develops turbidity it should be discarded.

Some reluctance has been expressed toward the employment of a standard and of a reagent containing cyanide. The concentration of cyanide in the reagent that is proposed for use is only 50 milligrams of potassium cyanide per liter. Its lethal dose for man approaches four liters and accidental aspiration of a few ml. during pipetting is of no consequence. Most clinical laboratories use for the determination of uric acid a reagent containing 50 grams of this salt per liter. In view of this, handling of the proposed reagent would seem a quite negligible hazard. However, care should be taken in handling solid KCN. While the concentration of cyanide in the diluent solution is insignificant, the salt itself is poisonous and should be handled only by highly responsible people.

In laboratories performing large numbers of cyanmethemoglobin determinations, it is desirable that the residues should be discarded into a free flow of water.

8. Blank

The diluent solution may be used as a blank when doing hemoglobinometry by the cyanmethemoglobin method. However, the absorption of light by this material is negligible at a wave length of 540 mμ so that it is possible to use distilled water as a blank instead.

USE OF THE STANDARD WITH COLEMAN, JR., SPECTROPHOTOMETER

This is an example of the way in which the standard is used with a photometric instrument.

A. Calibration of the Instrument with Cyanmethemoglobin Standards

1. Calibrate the instrument according to the manufacturer's instructions using the didymium glass filter. Place the standard solutions in a set of Coleman B cuvettes that have been matched. These cuvettes may be sealed for permanence. From the larger end of a No. 0 rubber stopper, cut a slice approximately 5 mm. thick. Drop this into the cuvette adapter in the well. This modification is necessary in order to make accurate measurements with only 5 ml. in the cuvettes.

2. Set the wave length scale at 540 mμ .

3. Turn the cuvette adapter 90^{o} to obstruct the light beam and adjust the galvanometer scale so that the galvanometer light beam rests on 0% Transmittancy (Black Scale). Return the cuvette adapter to its normal position and place in it a cuvette containing distilled water to serve as a blank. Using the coarse and fine adjustment knobs, bring the galvanometer light beam to rest on D "0" (Red Scale).

4. In turn, place the standard tubes in the cuvette well, noting the D reading for each. Use these values to construct a standard curve or standard table for clinical hemoglobinometry.

B. Preparation of a Standard Curve and a Standard Table for Hemoglobinometry

1. Hemoglobinometry in the Coleman, Jr., is carried out at a dilution of 0.02 ml. of blood in 5 ml. of diluent, representing a 251 fold dilution. The exact values of the standard solutions should be translated into terms of grams of hemoglobin per 100 ml. of blood. These values should be used in the construction of the standard curve below.

2. Obtain a piece of linear graph paper. The ordinate represents D; the abscissa is the hemoglobin in grams per cent. Plot the values obtained for the D reading of the standard solutions against their corresponding grams per cent of hemoglobin. A line connecting the points should pass through or very near zero. This is the standard curve. When unknown specimens of blood are measured, the D reading may be translated into values of hemoglobin by reference to this curve.

3. From this curve it is possible to construct a table of every possible D reading and its corresponding value in hemoglobin. Where large numbers of measurements are done, the table may be more convenient than the curve.

C. Hemoglobinometry by the Cyanmethemoglobin Method

1. With a volumetric pipet, measure exactly 5 ml. of the diluent solution in a clean dry cuvette.

2. Mix the sample of blood thoroughly and transfer exactly 0.02 ml. (Sahli pipet) of blood into the diluent solution. Rinse the pipet three times with the diluent in the cuvette. Mix the blood and the diluent thoroughly by swirling the tube.

3. Allow the tube to stand for 10 minutes before attempting photometric measurement. This allows cyanmethemoglobin to form.

4. Turn on the photometer. Set the wave length scale at 540 mμ and carry out the adjustments described in Par. A. (3) of the calibration instructions above.

5. Remove the blank from the cuvette well and replace it with the cuvette containing the unknown. Observe the D reading. From the standard curve or standard table obtain the hemoglobin value that is equivalent to the D reading.

Haden-Hausser Method

Discussion. N. B. This method is included only as a guide for use under field conditions.

A convenience of the Haden-Hausser method is the use of the blood specimen in the white blood pipet to make a white blood count prior to the hemoglobin determination.

There are eight common errors in technic:

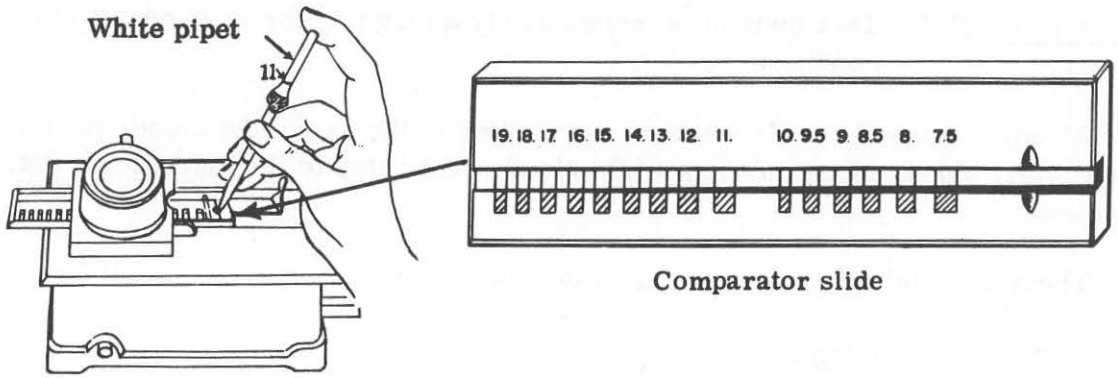
1. Dirty diluting fluid.
2. Dirty instruments.
3. Faulty and hasty readings.
4. Inaccurate pipeting.
5. Improper light.
6. Failure to discard the first drop of diluting fluid.
7. Failure to make the proper dilution.
8. The inherent difficulty in matching shade gradations between the standard comparator and the acid hematin solution.

The Haden-Hausser hemoglobinometer is illustrated on page 16, and consists of:

1. A series of illuminated rectangles separated by alternate dark spaces appear in the field of the reading lens.
2. The lower half of each rectangle constitutes the color standard, while the upper half of each rectangle consitutes the dilution when the wedge-shaped chamber is filled with the acid hematin solution.
3. A thin, uniform film of the acid hematin will extend by capillarity from the dilution chamber and cover the comparator color standard. This insures a light transmitting surface common to both the dilution channel and standard. The depth of the solution varies because of the wedge-shaped channel.
4. The scale, invisible during the process of color matching, is brought into the field by a shutter when the reading is made.

Procedure

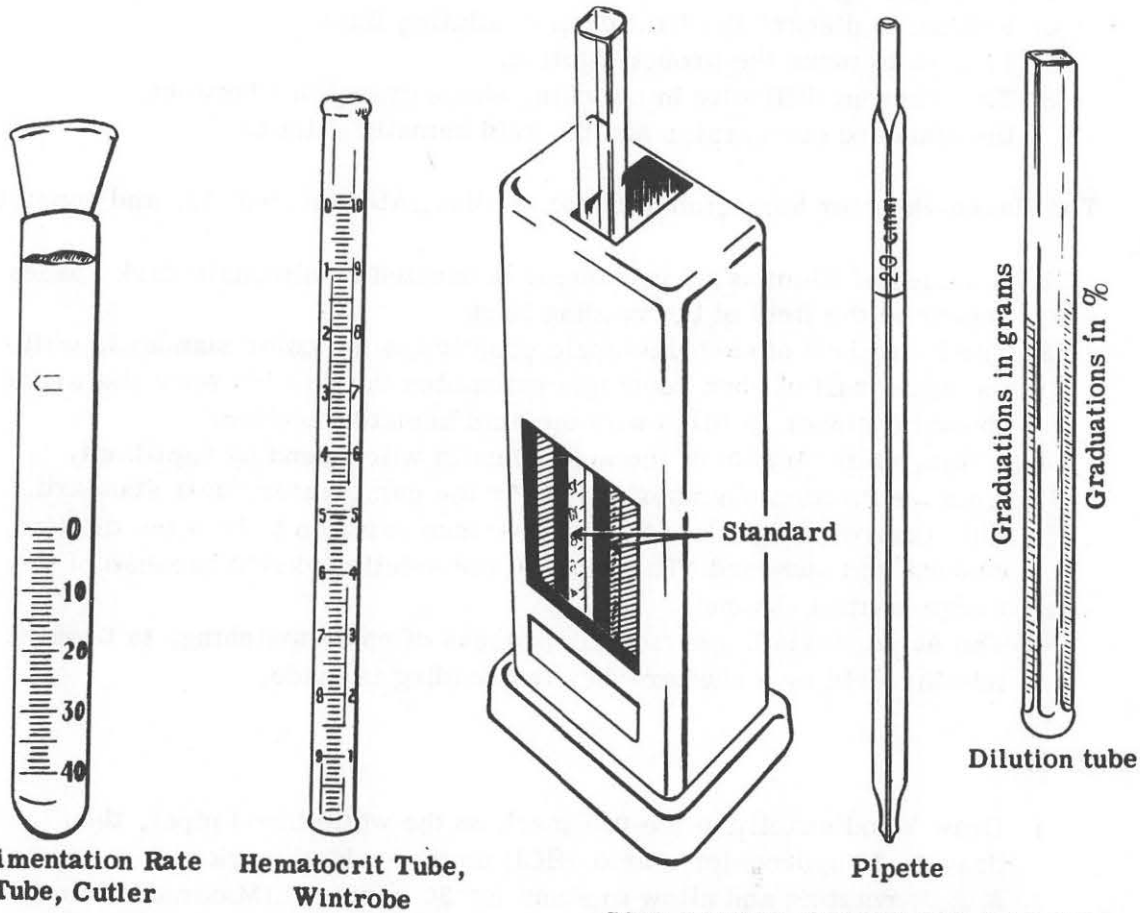
1. Draw blood exactly to the 0.5 mark on the white blood pipet, then draw N/10 hydrochloric acid (HCl) up to the 11.0 mark.
2. Mix by rotating and allow to stand for 30 minutes. (Maximum color will develop in this time, although 95 per cent of the color is developed in 10 minutes.)
3. Shake the pipet and discard the first 3 drops of fluid.
4. Discharge the contents of the pipet into the wedge-shaped channel of the



Comparator slide

Laboratory model

HADEN-HAUSSER HEMOGLOBINOMETER



Sedimentation Rate Tube, Cutler

Hematocrit Tube, Wintrobe

Pipette

Dilution tube

SAHLI TYPE HEMOGLOBINOMETER

comparator at the end of the coverglass. Caution - if WBC is wanted, fill hemocytometer first.

5. Move the comparator through the field until the proper match is made.

Note: When the hemoglobin is below 7.5 gms., blood is drawn to the 1.0 mark on the pipet and the reading is divided by two. Learn to estimate the hemoglobin as it appears in the pipet, and take a "double" pipet-full when it is low.

Diluting Solution

N/10 Hydrochloric Acid Solution

Concentrated hydrochloric acid .... 8.6 cc.
Distilled water, q.s. ..... 1,000 cc

Sahli Method

Procedure. N.B. Recommended for use under field conditions only.
The Sahli hemoglobinometer is illustrated on page 16.

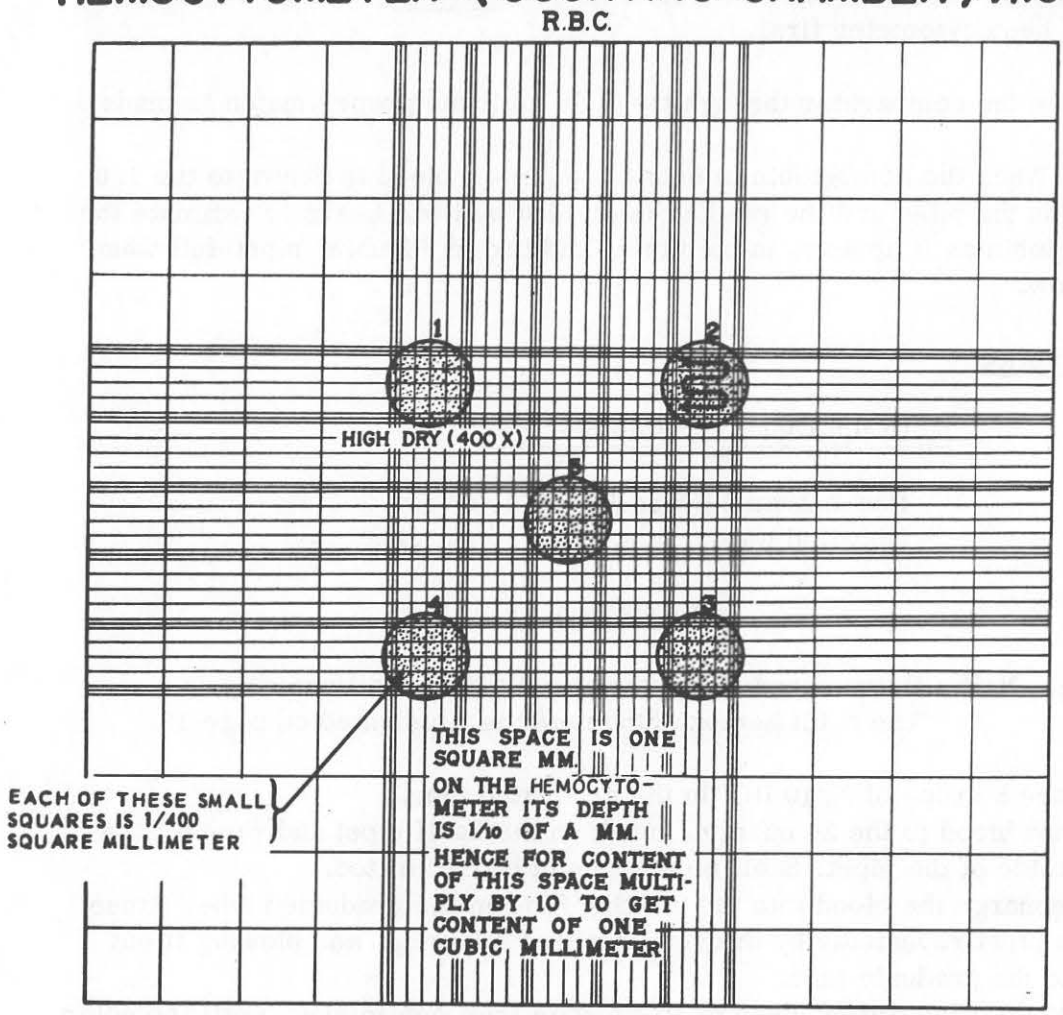
1. Place 5 drops of N/10 HCl in the graduated tube.
2. Draw blood to the 20 cu. mm. mark on the Sahli pipet and wipe off the outside of the pipet. Sahli pipets should be calibrated.
3. Discharge the blood into the diluting fluid in the graduated tube. Rinse the pipet repeatedly by drawing distilled water up, and blowing it out into the graduate tube.
4. Add distilled water, drop by drop, with thorough mixing, until the color matches the permanent standard. A standard light source is preferable. Be careful not to overdilute, for then the test must be started again.
5. The hemoglobin, in grams and percentage, is read from the sides of the tube at the height of the meniscus of the fluid. The reading should be made 5 minutes after dilution is started.

THE RED CELL COUNT

Discussion

Recent studies have shown that there is an overall inherent and technical error varying up to 25 per cent in the red cell count. Therefore extreme care is important in performing this test.

HEMOCYTOMETER (COUNTING CHAMBER) X193



IMPROVED NEUBAUER RULING

R.B.C. COUNT

There are nine common technical errors to avoid:

1. Dirty diluting fluid and apparatus. Change to fresh solution and clean all gear often.
2. Failure to wipe excess blood from the end of pipet when making the dilution and to recheck the blood column's meniscus after wiping.
3. Improper dilution (filling below or above mark).
4. Improper preparation of diluting fluid.
5. Failure to count, or recounting, cells near rulings on chamber.
6. Making count on flooded chamber.
7. Failure to mix properly and discard first three drops from pipet.
8. Use of wet or broken pipets or chambers.
9. Presence of air bubbles in chamber.

Pipets are useless if the ends are chipped, therefore care must always be taken to avoid chipping. Pipets should be cleaned first with water, then alcohol, acetone, and finally dried thoroughly. All procedures can be carried out on a suction apparatus. A dry pipet is one in which the bead is free in the bulb. Periodic cleaning with sulfuric acid-dichromate solution is recommended.

Equipment

The equipment consists of a red cell diluting pipet, a counting chamber, and red cell diluting fluid. The pipet is graduated so that dilutions up to 1/1000 can be made. Routinely, a 1/200 dilution is used and this is accomplished by filling the pipet with blood to the 0.5 mark and diluting up to the 101 mark with fluid.

Procedure

1. Fill the pipet exactly to the 0.5 mark with oxalated blood, or blood from a finger puncture.
2. Wipe excess blood from tip of pipet without disturbing the column of blood. Recheck the blood column.
3. Fill the pipet to the 101 mark with red diluting fluid.
4. Shake for 3 minutes. The pipet should not be shaken in the direction of the long axis.
5. Place cover slip on chamber.
6. Discard 1/3 of fluid in pipet.
7. Fill both sides of the chamber by lightly touching edge of cover slip and chamber with tip of pipet. If the fluid runs over the moat, start over again with clean, dry chamber.
8. Allow red cells to settle. This takes approximately one minute.
9. Place chamber on microscope and under low-power and subdued light locate the center square millimeter area. Switch to the high-dry power.
10. Count all the cells in 5 medium-sized squares. These areas are

surrounded by double or triple lines. It is well to count 5 widely separated areas; for example, the four corners and the center of the finely ruled area. In counting cells which lie upon the border lines, count those which touch the upper and left border lines and disregard those touching the lower and right border lines.

11. Multiply total cells counted by 10,000 (the red cell correction factor). The resulting figure will be the total count per cubic millimeter.

Calculations. Cf. illustration of counting chamber on page 184.

Red cell correction factor:

Each of the smallest squares equals 1/400 square millimeter in area. One triple-ruled medium square contains sixteen small squares. Five medium sized squares are counted; therefore, a total of 80 small squares are counted, or an area of 80/400 which equals 1/5 square millimeter. The counting chamber has a depth of 1/10 millimeter and the dilution is 1:200. The red cell correction factor 10,000 is obtained by multiplying 5 (to correct to 1 square millimeter) times 10 (to correct to 1 millimeter depth) times 200 (to correct for $\frac{1}{200}$ dilution.)

5,400,000 ± 800,000 red blood cells per cubic millimeter is normal for men; 4,800,000 ± 600,000 for women.

Diluting Solutions. These are designed to preserve red cells while destroying other cells.

Gower's Solution: Gower's solution is preferred as it prevents clumping of red cells.

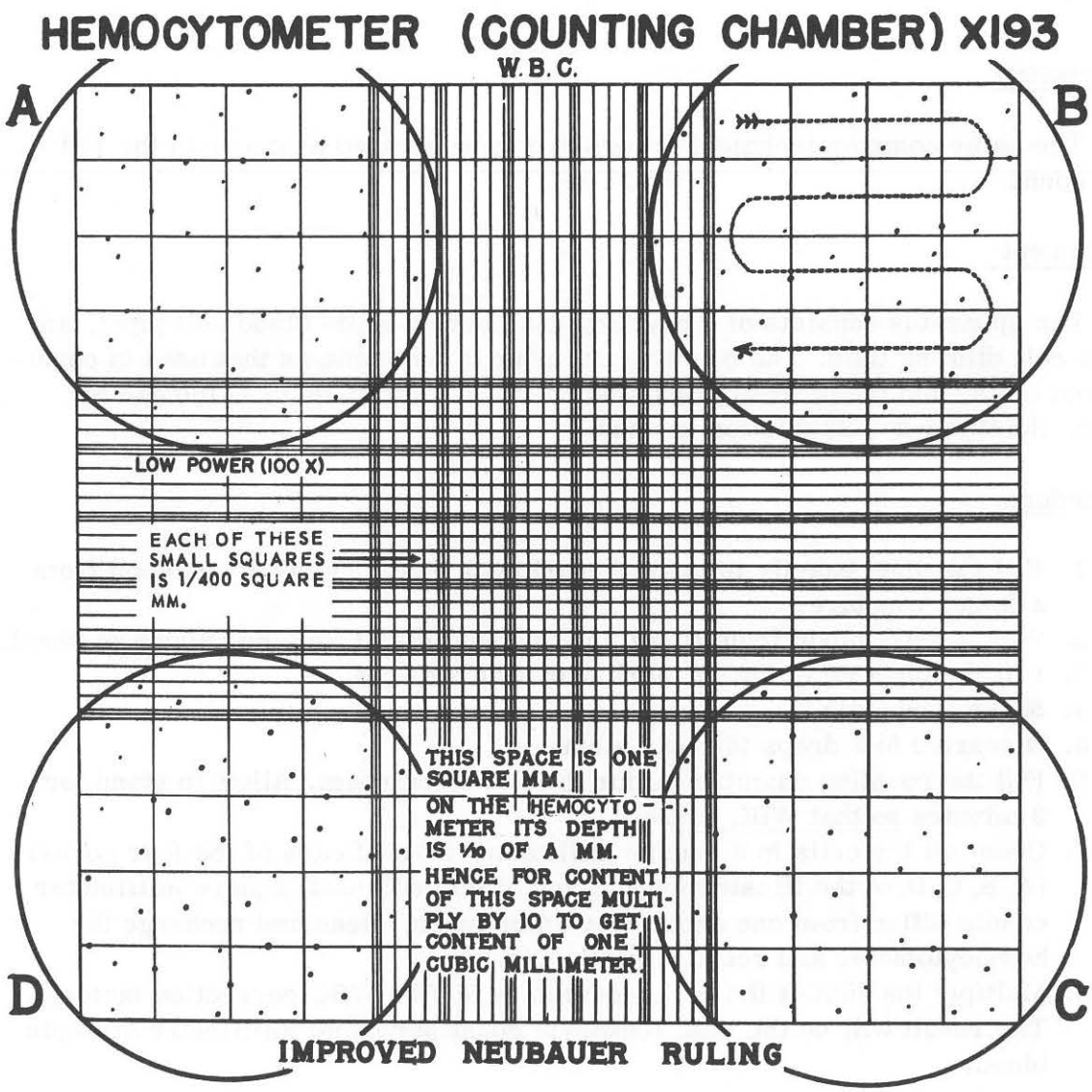
Sodium sulfate	6.25 gms.
Glacial acetic acid	16.665 ml.
Distilled water	100.00 ml.

Hayem's Solution:

Bichloride of mercury	0.5 gms.
Sodium sulfate	5.0 gms.
Sodium chloride	1.0 gms.
Distilled water	200.0 ml.

Toisson's Solution:

Sodium chloride	1.0 gms.
Sodium sulfate	8.0 gms.
Neutral glycerin	30.0 ml.
Distiled water	160.0 ml.


HEMOCYTOMETER (COUNTING CHAMBER) X193
W.B.C.
A
B
LOW POWER (100 X)
EACH OF THESE SMALL SQUARES IS 1/400 SQUARE MM.
THIS SPACE IS ONE SQUARE MM. ON THE HEMOCYTO-METER ITS DEPTH IS 1/10 OF A MM. HENCE FOR CONTENT OF THIS SPACE MULTI-PLY BY 10 TO GET CONTENT OF ONE CUBIC MILLIMETER.
D
C
IMPROVED NEUBAUER RULING

W.B.C. COUNT

THE WHITE CELL COUNT

Discussion

The same common technical errors are to be avoided as occur in the red cell count.

Equipment

The apparatus consists of a counting chamber, a white blood cell pipet, and white cell diluting fluid. The counting chamber is the same as that used in counting red cells, and the pipet is graduated so that a dilution up to 1/100 can be made. Routinely a 1/20 dilution is used.

Procedure:

1. Fill the pipet exactly to the 0.5 mark with oxalated blood, or blood from a finger puncture.
2. Wipe excess blood from tip of pipet without disturbing the column of blood.
3. Fill the pipet up to the 11 mark with diluting fluid.
4. Shake 2 minutes.
5. Discard 2 to 3 drops to clear stem.
6. Fill the counting chamber as for the red blood count. Allow to stand for 3 minutes so that WBC settle.
7. Count all the cells in a square millimeter area of each of the four corners (A, B, C, D, of the illustration). If any of the individual square millimeter counts differ from one another by 10 or more, clean and recharge the hemocytometer and recount the WBC's.
8. Multiply the sum of the cells counted by 50 (the WBC correction factor). The result will be the total leukocyte count per cubic millimeter of whole blood.

WBC Correction Factor

The original dilution was 1:20 and the depth of the chamber is 1/10 millimeter; the area of each square counted is one square millimeter and 4 square millimeters were counted.

$$\frac{20 \times 10 \times 1}{4} = \frac{200}{4} = 50$$

There are normally from 5,000 to 10,000 leukocytes per cubic millimeter of blood.

Note: White cell diluting fluids preserve nucleated cells and destroy others. When nucleated erythrocytes, as in erythroblastosis fetalis, are present in the blood they are preserved by the white cell diluting fluid and are indistinguishable from

lymphocytes in the counting chamber. When nucleated erythrocytes are present in excess of 10 per 100 leukocytes, as determined in the differential count of the stained smear, the total white count must be corrected. To make this correction calculate the percentage of WBC per 100 nucleated cells in the stained smear, then multiply the hemocytometer count by this percentage.

% WBC per 100 nucleated cells x Hemocytometer count = Corrected WBC count.

Diluting Solutions. (Designed to preserve nucleated cells and destroy others.)

Acetic Acid Solution:

Glacial acetic acid	3.0 ml.
Gentian violet, 1 per cent aqueous solution	1.0 ml.
Distilled water, q.s.	100.0 ml.

The gentian violet is not necessary, but it makes the cells more easily seen by staining the nucleus and thereby makes the count more accurate. When in use, make a practice of filtering this solution daily.

Hydrochloric Acid Solution:

N/10 HCl may be used as the white blood cell diluting fluid. This is convenient when using the Haden-Hausser hemoglobinometer because the white count and hemoglobin determination may be done on the same pipet. The white count must be done first. See previous directions on the preparation of N/10 hydrochloric acid solution. (cf page 17.)

THE DIFFERENTIAL WHITE BLOOD COUNT

Discussion

The enumeration of the percentages of the various white blood cells is called a differential white blood cell count. There are various systems of classification all of which depend upon the proper identification of the various cells and the recognition of immature forms of these cells. Routinely, the differential count is made according to SCHILLING'S CLASSIFICATION which enumerates the various blood cell percentages and, in addition, classifies the neutrophils according to their degree of maturity. The presence of immature neutrophils in the blood is called a "left shift."

In order to study the cells satisfactorily it is necessary to stain them. The aniline dyes which are extensively used in blood work are of two general classes, basic dyes of which methylene blue is an example, and acid dyes of which eosin is an example. Nuclei and certain other structures in the blood which are stained by

the basic dyes are called basophilic. Certain structures take up only acid dyes and are called acidophilic or eosinophilic. Certain other structures are stained by a combination of the two and are called neutrophilic.

Wright's stain is satisfactory and is used routinely at the U. S. Naval Medical School. In order to obtain a consistently superior stain it is necessary to properly prepare all materials and to establish a proper staining routine.

Procedure

Technic for making blood smear:

Whether coverglasses or slides are used, they must be clean, dry, and grease free. Preparations may be made from remaining blood in the syringe if done immediately. Coverglass preparations are preferred.

1. Place a small drop of blood on one coverglass and quickly place another coverglass on the drop.
2. Quickly pull the two coverglasses apart in a sliding fashion and rapidly air dry.
3. Repeat this procedure until two or more smears are prepared.

Technic of staining:

1. Apply a suitable holder, such as a Stewart forcep, to coverglass and cover with a measured quantity of Wright's stain - 8 drops for coverglass; 30 drops for a slide. Allow to fix for 1 minute. Then add an equal quantity of pH7 buffer solution.
2. Mix the buffer and the stain thoroughly by blowing gently on the mixture until a metallic scum forms on the surface. The staining time is usually 4 or 5 minutes. Flood the stain off the slide by washing with neutral wash water. (Hold the slide horizontal to prevent streaks.)
3. Dry in air. Mount in balsam or immersion oil and examine.
4. The staining time in the above procedure may vary with each batch of stain.

When polychrome methylene-blue-eosin stains are properly used, failure to get satisfactory results may be due to imperfect polychroming of the powder, but most frequently it is a question of incorrect reaction of the staining fluid. If the solution is too acid the erythrocytes stain bright red and the nuclei of the leukocytes are pale sky-blue or even colorless. When it is too alkaline the red corpuscles stain deep slate-blue and there is little differentiation of colors. In general, a preparation is satisfactory when both nuclei and neutrophilic granules are distinct and when the film is free of precipitated dye. Pathologic blood will sometimes not stain well with solutions which are correct for normal blood. This point should be remembered.

Preparation of Wright's Stain

The stain is prepared by dropping 0.3 gms. of certified Wright's powder on the surface of 100 cc. of recently opened, chemically pure, absolute methyl alcohol. Often the absolute methyl alcohol is too acid and requires neutralization as follows:

1. Add a few small crystals of hematoxylin to the methyl alcohol. If the alcohol is acid a yellow color will develop.
2. Dissolve small quantities of the buffer salt mixture (pH 6.8 or pH 7) in the methyl alcohol until the color changes to a faint pinkish lavender.
3. Filter the alcohol and use to prepare the Wright's stain.

Several batches are made and labeled with the date of manufacture. The batches are permitted to ripen in a dark place for at least 30 days. During this time, shake at intervals. At the end of 30 days filter the desired quantity of stain into a dropping bottle. Keep stock and working solutions free of water and well-corked to prevent evaporation.

Much of the trouble in staining with polychrome stain is eliminated if buffer solution is used instead of distilled water to dilute the stain. At this activity a buffer of pH 7 is preferred. This solution is prepared in the following manner:

Dibasic sodium phosphate ..... 5.447 gms.
Monobasic potassium phosphate ..... 4.752 gms.

Triturate the chemicals together to form the buffer salt mixture. This mixture is stable when dry and is well suited to work in the field. Add 1 gm. of the buffer mixture to 2,000 cc. of water. This solution has been checked with the potentiometer and has given consistent readings of pH 7.

Some stains may give more satisfactory results if buffered with a mixture having a pH of 6.8. The following formula is satisfactory for such a mixture:

Dibasic sodium phosphate ..... 4.539 gms.
Monobasic potassium phosphate ..... 5.940 gms.

Thoroughly mix the salts in a mortar and use 1 gm. to 2,000 cc. of water.

The wash water should be carefully checked by adding a pinch of hematoxylin crystals to 5 cc. of water. After shaking for two minutes, if the supernatant fluid is yellow, the wash water is acid and will act to decolorize the smear. The wash water may be neutralized by adding 1 gm. of the buffer salt (pH 7) mixture to 2,000 cc. of water.

Once a satisfactory technic has been established it should be used routinely.

Study of the Stained Blood Smear

Generally speaking, it is not only the increase in the number of white cells during the course of a disease that determines its severity. Some of the most severe diseases have a low total white count. The type of white blood cell found is also of diagnostic and prognostic importance. For this reason the study of stained blood smears is one of the most important functions of the technician.

The ability to recognize and differentiate the various cells requires a thorough knowledge of their morphologic characteristics and of their staining qualities. Considerable supervised practice is required.

A study of well prepared specimens of normal bone marrow and of blood smears of the leukemias offers a satisfactory means by which the beginner may familiarize himself with the various types of immature cells. In addition to the differential count, a great deal of useful information can be gained by the careful examination of a blood smear (that is, by noting the relative number and size of thrombocytes; the size, shape, and hemoglobin content of the red blood cells; the presence of polychromatophilia which, if marked, is indicative of increased activity of the erythrocytic series; the presence of blood parasites; and the presence of basophilic stippling). Report any abnormalities noted.

In addition to enumerating the types of cells present during the differential count, one should observe the relative size of the white blood cell, the presence of toxic neutrophils, the presence of hypersegmentation, etc. There should be no attempt to classify degenerating cells, but their presence should be noted.

The distinction between metamyelocyte and band (or between band and segmented neutrophil) is sometimes difficult to make. The rule is to classify the cell as the more mature form. Several areas of the slide should be examined including both the center and the edges of the smear. Counting 200 cells and dividing by 2 gives a more accurate differential count than counting 100 cells. Be certain the count totals 100 per cent.

The routine differential examination should first include a study with the low-power objective to check the staining and distribution of cells. An estimate of the total white count under low-power should check roughly with the wet count. The high-dry objective should then be used and the slide examined for abnormal cells and malarial and filarial parasites. Finally, using an oil-immersion objective, classify 100 white cells. If the count does not fall within a normal range, it is well to classify 200 cells or more. When very few leukocytes are present, it is of value to make a smear from the concentrated leukocyte layer in the hematocrit tube and to use this for the differential count.

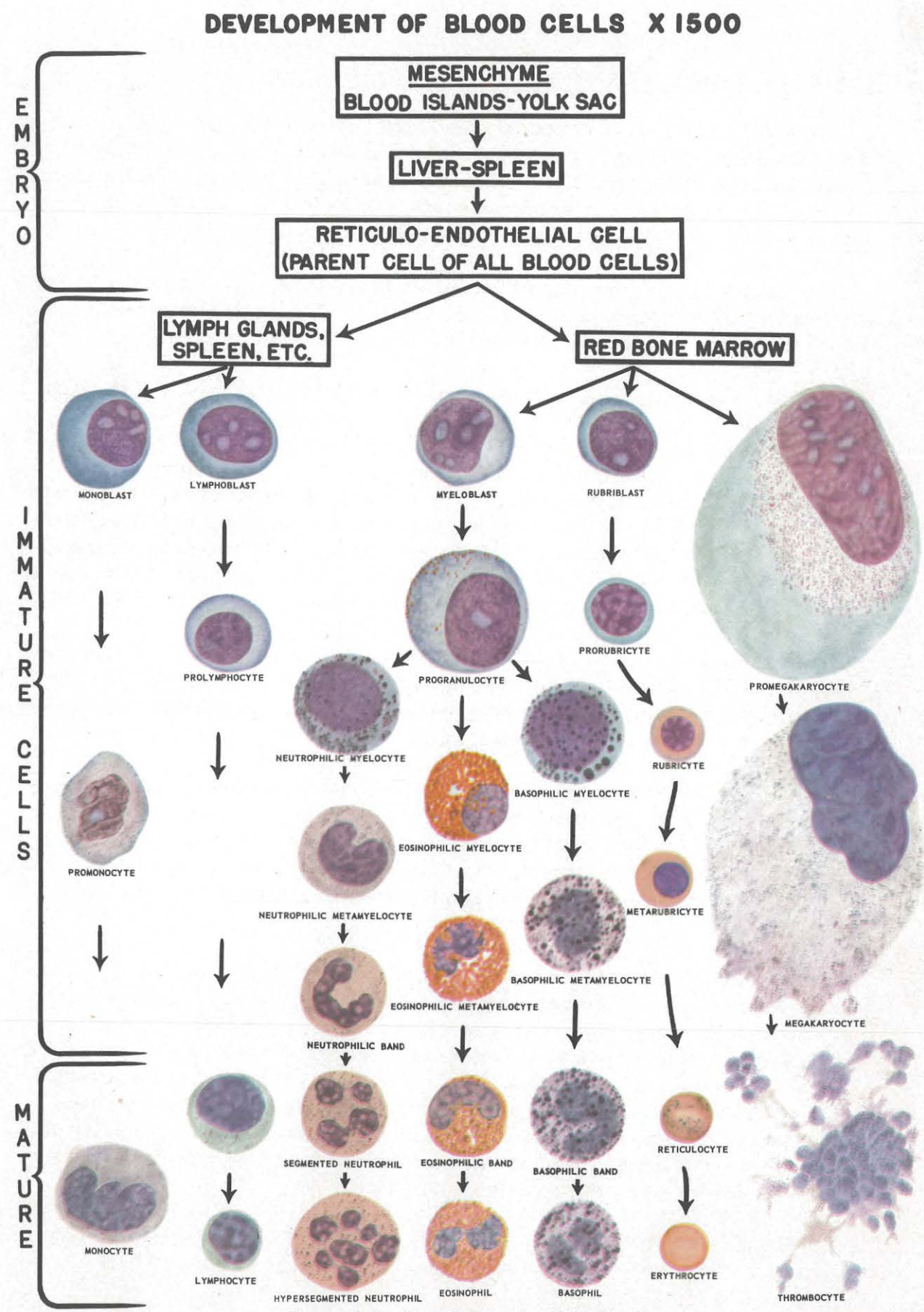
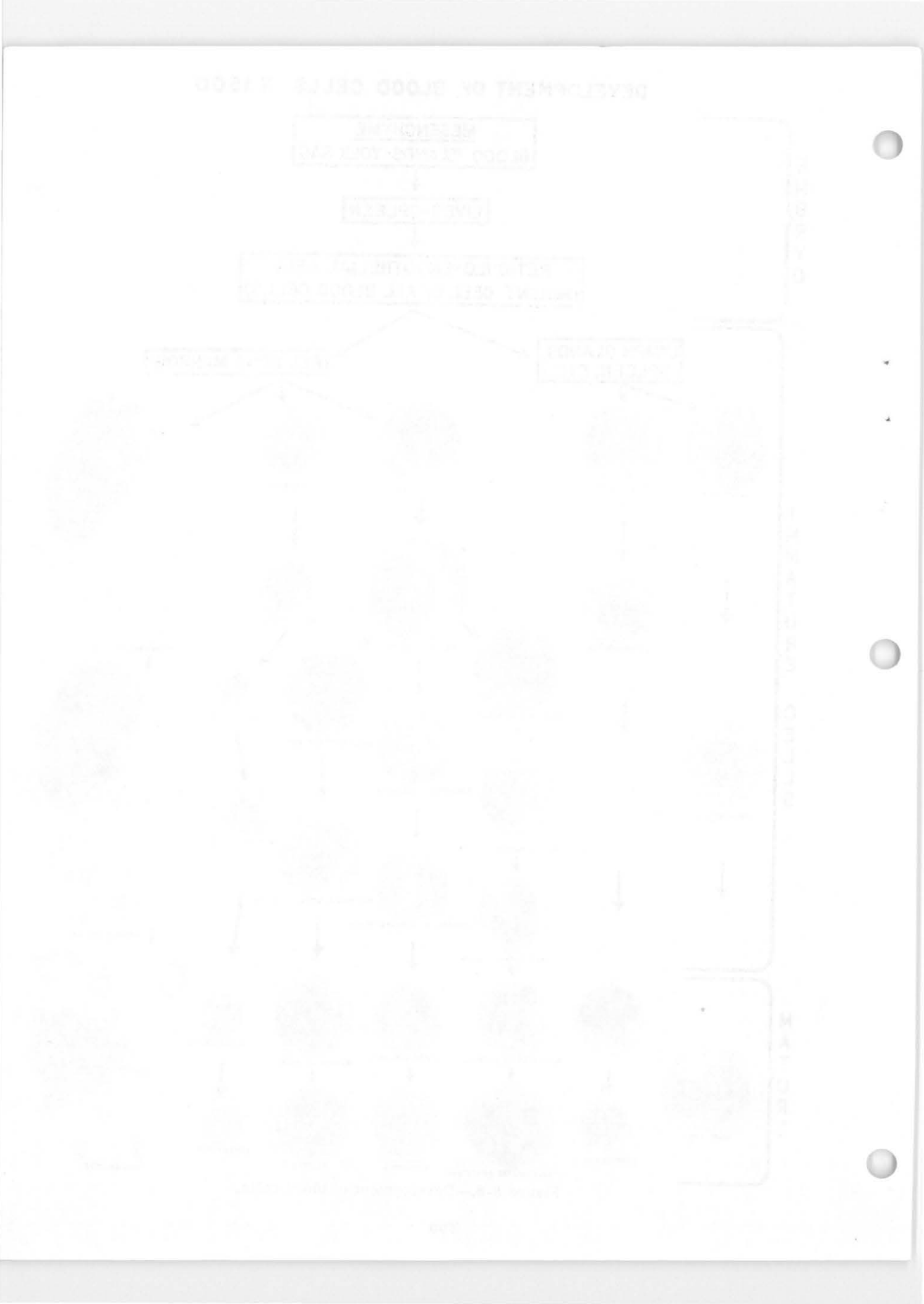


Figure 8-8.—Development of blood cells.



ORIGIN OF BLOOD CELLS

There are many theories concerning the production and growth of the various blood cells. Hematologists are in accord in only one concept, that all blood cells have their origin from the mesenchymal cell of the mesodermal layer in the embryo. Beyond this the theories vary considerably.

Pappenheim and Maximow are considered the founders of the monophyletic theory. They believe that in adult life all types of blood cells have their common origin in one primitive stem cell.

Ehrlich advanced the polyphyletic or dualistic theory. He believed that the common ancestral cell is not present after birth, but that in postnatal life there are two parent cells the myeloblst, giving rise to the monocytes, granulocytes and erythrocytes; and the lymphoblast, giving rise to the lymphocytes.

NOMENCLATURE

The following suggestions made by the committee for the clarification of the nomenclature of cells, and diseases of the blood and blood-forming organs, sponsored by the American Society of Clinical Pathologists and the American Medical Association, are reproduced for reference here. Their use is recommended.

It is also recommended that the term leukocyte be considered synonymous with white blood corpuscle. This term includes all cells of the granulocytic, lymphocytic, monocytic and plasmacytic series. This and other words derived from the same root should be spelled with a k and not a c, e.g., leukocyte, leukemia, not leucocyte or leucemia.

TABLE 5.

RECOMMENDED TERMS, AND TERMS TO BE AVOIDED, WHEN REFERRING TO CELLS OF A PARTICULAR SERIES OR TO A DISEASE AFFECTING ANY CELL OF THAT SERIES

Term to be Used	Terms to be Avoided
Lymphocytic	Lymphoid, lymphatic, lymphogenous lymphocyte, mononuclear
Granulocytic	Myeloid, myelogenous, myelocyte, myelocytic granulocyte, leukocyte, leukocytic, leucocyte, leucocytic

(cont.)

Term to be Used	Terms to be Avoided
Monocytic	Monocytoid, monocytogenous, mononuclear, monocyte
Plasmacytic	Plasma cellular, plasmacytogenous, myeloma cell, plasmacyte
Erythrocytic	Erythroid, erythrocytoid, erythron, erythrocytogenous, erythrocyte
Thrombocytic	Megakaryocytic, platelet, thrombocyte

It is recommended that the descriptive terms for granules, i.e., neutrophil, eosinophil, basophil, and azurophil, be spelled as indicated without a final "e".

It is suggested that the name of the most undifferentiated of the cells of each series carry the suffix "blast;" the second stage, the prefix "pro" and, except in the granulocytic series, all cells that are more mature than the "pro" stage have names with the suffix "cyte." The name for the fourth stage in the granulocytic and erythrocytic series should have the prefix "meta." The terms "blast cells" and "pro cells" may be used to replace other terms for these stages of development when speaking of the stage of development as a whole, or when the series to which the cells belong has not been identified.

It is recognized that the blast cells of each series are morphologically very similar, all having fine nuclear chromatin, usually demonstrable nucleoli, and basophilic cytoplasm, with or without azurophil granules. The prefix to be used will, in many instances, depend on the identification of the "pro" stage associated with them.

Fine nuclear chromatin has a finely stippled, or lacy meshwork pattern. No aggregates of chromatin form even a single clump of appreciable size staining darker than other areas in the nucleus. Pale staining parachromatin forms a homogenous nuclear background.

A nucleolus is a distinct, ovoid, paler staining, intra-nuclear structure. It is usually pale blue with Wright's stain, but color may vary depending on the stain used.

The term azurophil should be applied to the granules seen typically in the cytoplasm of cells of the lymphocytic and monocytic series and in the progranulocyte stage of the granulocytic series. The term azurophil, not azure, is recommended when describing these granules, since the term refers to an affinity for a particular dye, and not to the inherent color of the granules. These granules may be present or absent in any cell of the lymphocytic series, and when

present are usually coarse and clumped. They are also usually present in cells of the monocytic series where they are fine, being diffusely and uniformly scattered through the cytoplasm. If not seen in the monocyte or promonocyte, it usually indicates a faulty stain or poor optical resolution by the microscope. These granules may be present or absent in any cell of the granulocytic series. They are rarely seen beyond the myelocyte stage except in disease. They are occasionally present in the cytoplasm of cells of the plasmacytic series; they are constantly present in the cells beyond the blast stage in the thrombocytic series where they tend to be fine and few in the early stages, and numerous and often clumped in the more mature stages.

It is recognized that in each cell series there is a continuous development from the most undifferentiated to the most differentiated stage. Therefore cells will be encountered where recognition is difficult, in which case it is suggested that the cell be arbitrarily placed in the more differentiated category.

The formed elements normally in peripheral blood are derived from a universal, multipotent, parent stem cell, the reticulum cell, which is found in the bone marrow, liver, spleen, lymph nodes, and as adventitial cells about blood vessels in all other organs. In smears, they are 20-30μ diameter with abundant pale basophilic cytoplasm, occasionally azurophil granules. The nucleus is large, round or ovoid and has pale staining chromatin forming a fine mesh. One or more nucleoli are present. As it matures, the cytoplasm becomes more basophilic.

TABLE 6.

Name of Series	Term to be Used	Terms to be Avoided
Lymphocytic	Lymphoblast	Myeloblast, hemocytoblast, lymphoidocyte, stem cell, lymphocyte
	Prolymphocyte	Large lymphocyte, pathologic large lymphocyte, atypical leukocytoid lymphocyte, monocyte, immature lymphocyte
	Lymphocyte	Small, medium or large lymphocyte, normal lymphocyte, small, medium or large mononuclear.
Monocytic	Monoblast	Myeloblast, hemocytoblast, lymphoidocyte, lymphocyte, stem cell, immature monocyte

(cont.)

Name of Series	Term to be Used	Terms to be Avoided
Monocytic (cont.)	Promonocyte	Premonocyte, hemohistioblast, immature monocyte, Ferrata cell
	Monocyte	Large mononuclear, transitional, clasmatocyte, endothelial leukocyte, histiocyte, resting wandering cell
Granulocytic	Myeloblast	Granuloblast, hemocytoblast, lymphoidocyte, lymphocyte, stem cell
	Progranulocyte	Promyelocyte II, leukoblast, myeloblast, premyelocyte, progranulocyte-A
	Myelocyte	Granulocyte, myelocyte B, non-filament class I
	Metamyelocyte	Metagranulocyte, juvenile, myelocyte C, non-filament, class I
	Band cell	Staff cell, stab cell, non-filament, class I, rod nuclear, polymorphonuclear, stab-kernige, rhabdocyte, non-segmented
	Segmented	Polymorphonuclear, filamented, class II, III, IV, or V, lobocyte
Plasmacytic	Plasmablast	Myeloblast, hemocytoblast, lymphoidocyte, lymphocyte, stem cell, lymphoblastic plasma cell, myeloma cell
	Proplasmacyte	Turk cell, Turk irritation form, lymphoblastic or myeloblastic plasma cell, myeloma cell
	Plasmacyte	Plasma cell, Unna's plasma cell, Marschalko's plasma cell, plasmacytoid lymphocyte, myeloma cell

(cont.)

Name of Series	Term to be Used	Terms to be Avoided
Thrombocytic	Megakaryoblast	Megalokaryoblast
	Promegakaryocyte	Premegalokaryocyte
	Megakaryocyte	Megalokarocyte
	Thrombocyte	Platelet, thromboplastid
	Disintegrated cell	Senile cell, smudge, basket cell, smear cell, degenerated cell

THE LEUKOCYTIC SERIES (Wright's Stain)

Granulocytic Series

Myeloblast: 10-20μ diam.

This blast cell of the granulocytic series has a round or oval nucleus about two-thirds the size of the cell. The nuclear membrane is indistinct and the nucleus may seem to fuse with the cytoplasm. The chromatin is finely reticular and sieve-like. The nucleoli are more or less indistinct and are usually two or more in number. The cytoplasm is a deep, clear blue, and contains no granules. Differentiation of the various blast cells is difficult, so they are usually identified by the "company they keep," i.e., the immediately adjacent cells.

Progranulocyte: 14-20μ diam.

The nucleus is oval or kidney-shaped. The chromatin is coarse. Nucleoli are less well-defined and fewer in number. The cytoplasm stains less deeply blue and coarse azurophilic granules appear in the cytoplasm.

Myelocyte: 10-18μ diam.

It is smaller, and the nucleus is round or oval. The chromatin is coarse and uneven. Nucleoli are not present. The cytoplasm is pale lilac. This is the first cell of the granulocytic series to contain specific staining granules in the cytoplasm. The type of granule may be either neutrophilic (fine, purplish pink), eosinophilic (coarse, red), or basophilic (coarse, dark blue). This and all subsequent cells of the granulocytic series should be characterized as neutrophil, eosinophil, or basophil.

Metamyelocyte: 10-18μ diam.

The nucleus is indented, oval-shaped and resembles a bean or kidney. The cytoplasm is pinkish-lilac and contains specific granules. The chromatin is coarse, and nucleoli are not present.

Band cell: 10-15 μ diam.

The nucleus is a curved or coiled band or rod which may be nodular or indented. No matter how marked the nuclear indentation may be, it is a band if it does not completely segment the nucleus into lobes connected by a filament. The chromatin is dense and clumped. The cytoplasm is pink and contains specific granules.

Segmented: 10-15 μ diam.

Any cell containing specific granules in which the lobes of the nucleus are connected by a filament. There are usually two to five lobes. A filament is defined as a threadlike structure. The chromatin is coarse and dense. The cytoplasm is pink.

Hypersegmented neutrophil: 10-20 μ diam.

It is somewhat larger than the segmented neutrophil and the granules are coarser and take a brighter stain. The nucleus may contain six to twelve lobes, and the lobes may be distinct or they may be of the "ribbon form" in which they are elongated lobes. These cells are usually seen in pernicious anemia.

Eosinophil: 10-15 μ diam.

This cell resembles the neutrophil except for the large, bright reddish-orange granules in the cytoplasm. It is usually not important to classify this cell on the basis of immaturity. The granules are spherical, uniform in size, and usually equally distributed throughout the cytoplasm. Rarely do the granules extend over the nucleus. The nucleus seldom contains more than three lobes.

Basophil: 10-15 μ diam.

This cell resembles the neutrophil except for the coarse, bluish-black granules which are unevenly distributed in the cytoplasm, and vary in size and shape. They often obscure the nucleus. It is usually not important to classify these cells on the basis of maturity.

Lymphocytic Series

Lymphoblast: 10-18 μ diam.

This is the blast cell of the lymphocytic series. The cytoplasm stains blue and is without granules. The nucleus is round or oval and lightly staining with one or two nucleoli. The chromatin is arranged in a fine network.

Prolymphocyte: 10-18 μ diam.

The nucleus is round with coarser chromatin than that of the lymphoblast and usually the nucleoli is single. The cytoplasm is a lighter blue and may contain azurophilic granules.

Lymphocyte: 6-18 μ diam.

Lymphocytes may be small (6-10), medium (10-14) or large (14-18), The nucleus is round or slightly indented. The chromatin is clumped, and deeply staining, becoming denser as cell size decreases. The cytoplasm varies in quantity, being scanty in small cells, and abundant in large ones. The cytoplasm is normally sky-blue and clear. Granules are azurophilic, when present, and most commonly are seen in the large cells.

Monocytic Series

Monoblast: 14-18 μ diam.

The nucleus is round or oval, and the chromatin is fine and has a spongy appearance. One or two nucleoli are present. The cytoplasm is a deep blue and nongranular.

Promonocyte: 14-18 μ diam.

The nucleus may be round, indented, grooved or folded. The chromatin is distributed in a fine meshwork and may contain a nucleolus. The cytoplasm is abundant and stains gray-blue and may contain fine azurophilic granules.

Monocyte: 12-18 μ diam.

This is the largest mature leukocyte found in the peripheral blood. The nucleus is large with foldings or deep indentations. The chromatin is in loose meshed strands and small clumps with no nucleolus. The nucleus assumes many shapes, from kidney-shaped to lobulated. The cytoplasm is abundant and appears opaque or muddy blue due to the presence of numerous fine, azurophilic granules. Overstaining may produce large purple granules suggestive of a metamyelocyte, in which case the nucleus remains the differentiating characteristic.

Plasmacytic Series

Plasmablast: 15-25 μ diam.

The chromatin is finely dispersed. The cytoplasm is scant and tends to be more opaque in staining than in the other leukocytic blast cells. The nucleus contains three to six poorly defined nucleoli.

Proplasmacyte: 15-25 μ diam.

The nucleus is round, and the chromatin is coarser than the blast. It contains one or more distinct nucleoli. The cytoplasm is more abundant and is very deep blue.

Plasmacyte: 10-20 μ diam. (Plasma cell)

The nucleus is relatively small and round and is usually eccentrically located. The chromatin structure is coarse and dense and may have the so-called "cartwheel" appearance. No nucleoli are present. The cytoplasm is a deep blue.

Thrombocytic Series

Megakaryoblast: 25-35 μ diam.

This blast cell is usually larger than any of the other blasts. The nucleus has a fine chromatin structure containing 2 to 6 nucleoli. The nucleus tends to be lobulated. The cytoplasm is basophilic and relatively sparse.

Promegakaryocyte: 25-50 μ diam.

The nucleus has a coarser chromatin structure than the blast and contains 0-2 nucleoli. It is less lobulated than the blast. Fine azurophilc granules are usually diffusely scattered through the cytoplasm.

Megakaryocyte: 40-100 μ diam.

The nucleus is usually irregularly lobular and the chromatin is coarse and clumped. Nucleoli are not evident. The cytoplasm contains many azurophil granules which are often clumped. The megakaryocytes and the promegakaryocytes are typically much larger than any other cell found in the bone marrow. Psuedopodal cytoplasmic projections at the periphery of the cytoplasm indicate platelet formation.

Thrombocytes: 2-4 μ diam. (Platelets)

It is a cytoplasmic fragmentation of the megakaryocyte. It does not contain a nucleus. Red or purple granules are usually centrally located and surrounded by clear cytoplasm.

A SYSTEM FOR CELL IDENTIFICATION

The first essential to accurate identification of the cells of the blood or marrow is a satisfactory smear and stain. A thick, poorly stained smear is useless.

The following system has been developed to permit identification of cells encountered in a properly stained preparation (Wright's stain). It is based on answering a series of simple questions.

I. What is the size of the cell? Is it:

1. Small as an erythrocyte? (7.5μ)
2. Medium as a segmented neutrophil? (15μ)
3. Large as a monocyte? (20μ)

II. What is the color of the cytoplasm? Is it:

1. Clear, pale sky-blue as a lymphocyte?
2. Hazy, gray-blue as a monocyte?
3. Pink, as a neutrophil?
4. Deeply basophilic as in blast cells and plasmacytes?

III. Are there any granules in the cytoplasm? If so, are they:

1. Azurophilic, fine and diffuse as in the monocyte?
2. Azurophilic and coarse as in the lymphocyte and progranulocyte?
3. Lilac-colored as in the neutrophil?
4. Orange-red and coarse as in the eosinophil?
4. Purplish-black, coarse and irregular as in the basophil?

IV. What are the characteristics of the nucleus? Is it:

1. Large, folded or irregular as in the monocyte?
2. Relatively large, round and dense as in the lymphocyte?
3. Relatively large, pale, with fine chromatin network containing nucleoli as in the blasts and "pro" cells?
4. Indented or kidney-shaped as in the metamyelocyte?
5. Horseshoe-shaped and condensed as in the band?
6. Lobed and condensed as in the segmented?

Peroxidase Stain (Sato-Sekiya)

This stain is valuable in the differentiation of immature cells of the granulocytic series from those of the lymphocytic series; therefore we tend to depend on it to tell us to which series the cells belong.

Reagents Used in Preparing Peroxidase Stain

Solution No. 1. 0.5 per cent aqueous solution of copper sulfate.

Solution No. 2. Benzidine solution: Rub 0.2 gms. of reagent benzidine base in a mortar with a few drops of water. Add 200 ml. of distilled water and filter. To the filtrate add 0.25 ml. of 3 per cent hydrogen peroxide. Place in tightly stoppered, dark-brown bottles. This solution will keep for several months. When it develops a brown color and forms a precipitate it should be discarded.

Solution No. 3. 1 per cent aqueous solution of safranin.

Technic.

1. Flood a fresh, dry blood smear with solution No. 1 for a few seconds and pour off excess.
2. Flood with solution No. 2 for 2 minutes. Wash with distilled water.
3. Flood with solution No. 3 for 2 minutes.
4. Wash with distilled water for 30 seconds. Dry and examine with oil-immersion lens.

Peroxidase positive granules present in the granulocytic series except the myeloblast, stain bluish-green. Basophilic granules usually do not take the stain. Blast cells and all lymphocytes are negative. Monocytes may contain a few peroxidase-positive granules. Toxic granules in neutrophils fail to stain.

The Range of Normal Leukocyte Counts, Adults (per mm^3)

Type of Cell	Per cent	Absolute Number		
		Average	Range	
			Minimum	Maximum
Total leukocytes	---	7,500	5,000	10,000
Myelocytes	0	0	0	0
Neutrophilic metamyelocytes	0 - 1	70	50	100
Band neutrophils	0 - 5	350	250	500
Segmented neutrophils	50 - 70	4,500	2,500	6,800
Lymphocytes	20 - 40	2,000	1,000	3,500
Eosinophils	0 - 3	150	25	300
Basophils	0 - 1	70	25	100
Monocytes	2 - 8	400	100	800

Note: To obtain the absolute number of any type of cell, multiply the total leukocyte count by the percentage of the cell in question. The percentage of each cell is the relative number of each cell type present per one hundred leukocytes counted during the differential stained slide examination.

Leukocytic Response to Disease:

Stress on local or generalized areas of the body often causes formation of increased numbers of leukocytes. Thus an acute bacterial infection usually causes a rise in the neutrophils. This is reflected in an elevation of the total leukocyte count, due to increased numbers of segmented, band, and occasionally metamyelocyte forms. Similar responses are seen in many other acute illnesses such as: myocardial infarction, burns, severe emotional stress, intestinal obstruction, some of the virus diseases as poliomyelitis, etc. The rise in neutrophils in these conditions is usually accompanied by a rise in the total leukocyte count since the other leukocytes remain at, or near, their original levels. When the disease process is very severe this may reach high levels (20,000-40,000/mm^3) with the appearance of more metamyelocytes and some myelocytes in the peripheral blood (Schilling's "shift to the left"). The leukocytes then mimic a leukemia pattern in the peripheral blood, and this situation is termed a leukemoid reaction. An elevation of the total white cell count may be seen in dehydrated patients secondary to poor fluid intake or excessive fluid loss as a result of burns, vomiting,

diarrhea, heat prostration, etc. As the acute disease process causing a neutrophilic stimulus is brought under control, a gradually increasing monocyte count may be noted.

As the body tends to heal the area subjected to injury, a slow rise in lymphocytes may occur. Common causes of lymphocytosis are certain chronic illnesses and many of the viral diseases. Viral diseases may be accompanied by a decrease in circulating neutrophils and some cause vacuolation of the lymphocyte's cytoplasm. Infectious mononucleosis is a viral disease which causes atypical forms of lymphocytes to be seen in the peripheral blood. At times this may be confused with lymphocytic leukemia.

Increased monocytes are frequently seen in tuberculosis and monocytic leukemia, subacute bacterial endocarditis, brucellosis, typhoid, rickettsial disease, Hodgkins disease, trypanosomiasis, Kala-azar and Gaucher's disease.

A rise in eosinophils often accompanies parasitic infections, severe allergies, and the early recovery phase of many severe infections.

Generally speaking, the severity of acute pyogenic infections may be estimated by the degree of change in the leukocytes of the peripheral blood as shown in the total white cell count and the differential count. These changes in peripheral leukocytes usually reflect a similar change in the bone marrow where the granulocytes, erythrocytes, and platelets are formed. High white cell counts usually indicate a good response by body defenses to the disease process. Increasing numbers of immature cells indicate a more severe disease process and perhaps one to which body resistance is failing, the latter being particularly true when accompanied by a falling total white cell count. The appearance of increasing numbers of eosinophils and monocytes usually indicates a process of recovery from the disease.

Toxic changes in neutrophils are helpful in following acute disease processes in that the more severe the toxic changes, the more severe is the illness. Toxic changes of neutrophils include: (a) basophilia (blue staining of the cytoplasm), (b) presence of cytoplasmic vacuoles, (c) presence of dark blue staining granules (toxic granules), (d) condensation of the nucleus and (e) inclusion (Döhle) bodies. These changes may be graded from one to four plus in intensity.

LEUKOCYTE FINDINGS IN SOME COMMON DISEASES

A. Neutrophilic leukocytosis

Acidosis
Acute hemorrhage
Convulsions
Erythremia
Generalized pyogenic infections
Infarctions
Localized pyogenic infections
Granulocytic leukemia
Rheumatic fever
Scarlet fever
Severe burns
Strenuous exercise
Sudden hemolysis of blood
The newborn

B. Eosinophilia

Allergic conditions
Chorea
Chronic granulocytic leukemia
Helminthic infestations
Polyarteritis nodosa
Scarlet fever
Tropical eosinophilia

C. Monocytosis

Active tuberculosis
Brucellosis
Monocytic leukemia
Protozoal infections
Subacute bacterial endocarditis

D. Lymphocytosis

Congenital syphilis
German measles (plasmacytes)
Healing tuberculosis
Infectious lymphocytosis
Infectious mononucleosis
Lymphocytic leukemia
Mumps
Whooping cough

E. Neutrophilic leukopenia

Agranulocytosis
Aplastic anemia
Banti's disease
Many virus diseases
Pernicious anemia
Primary splenic neutropenia
Protozoal infections
Malnutrition
Some toxic drugs & chemicals

PHYSIOLOGICAL LEUKOCYTOSIS

A physiological leukocytosis of 10,000 - 15,000 is frequently seen in the following conditions:

In the newborn
During late pregnancy
During labor
In a moribund condition
Following cold baths
Following exercise
Following meals
Accompanying severe pain

Usually the count is not over 15,000, but it is sometimes higher.

THE ERYTHROCYTIC SERIES (Red Blood Cells)

TABLE 7

Terms Used When Referring to Specific Cells of the Erythrocytic Series

New Terminology	Old Terminology	Abnormal Erythropoiesis
Rubriblast	Pronormoblast, proerythroblast	Promegaloblast
Prorubricyte	Basophilic normoblast, erythroblast (basophilic)	Basophilic megaloblast
Rubricyte	Polychromatophilic normoblast, intermediate erythroblast (polychromatophilic)	Polychromatophilic megaloblast
Metarubricyte	Late erythroblast (acidophilic) acidophilic normoblast, normoblast, orthochromic normoblast	Acidophilic megaloblast
Reticulocyte	Reticulocyte	Reticulated megalocyte
Erythrocyte	Erythrocyte	Megalocyte

The new terminology for the abnormal erythropoiesis associated with the macrocytic anemias is the new terminology as above with the qualifying phrase, pernicious anemia type.

Rubriblast: 14-19μ diam.

The nucleus may be round or oval. The chromatin forms a dense net which is coarser than that of the myeloblast. The nuclear membrane is thin, and two or more nucleoli may be found. The cytoplasm is non-granular, deeply basophilic (much more so than other blast cells), and small in quantity.

Prorubricyte: 10-15μ diam.

The nucleus is round but smaller than that of the rubriblast. The chromatin network is more compact and coarser. Nucleoli are seldom present. The cytoplasm is a brighter blue and more plentiful than that of the rubriblast.

Rubricyte: 8-12μ diam.

The nucleus is smaller in size, being slightly larger than half the diameter

of the cell; it has a thick nuclear membrane. The chromatin is clumped and may have a tendency to be arranged in radiating strands like the spokes of a wheel. The cytoplasm may be either blue or pink or mottled. The pink shading is due to the formation of hemoglobin.

Metarubricyte: 7-10μ diam.

The nucleus is very small and is less than one-half the diameter of the cell. The chromatin is markedly clumped, deeply basophilic and tends to stain uniformly without a trace of internal structure. The cytoplasm is orange-pink due to the increased amount of hemoglobin; a basophilic cast to the cytoplasmic coloration is referred to as polychromasia. The nucleus may be fragmented or in the process of extrusion.

Reticulocyte: 7-9μ diam.

It is slightly larger than the erythrocyte and has no nucleus. The cytoplasm is usually orange-pink, sometimes polychromatophilic. By means of special vital staining, bluish skein-like reticulum fragments can be seen in the cytoplsm. Normally, 0.5 to 1.5 per cent reticulocytes are present. Increased numbers are reliable evidence of increased rate of erythrocyte formation.

Erythrocyte: 6-9μ diam.

This is the mature red blood cell without a nucleus. It stains a salmon pink with a pale central portion because it is a biconcave disc. The average diameter is 7.5 microns.

Abnormalities of Erythrocytes in Quality and Quantity

Anisocytosis:

Variation in size (microcytes less than 6μ diam., macrocytes greater than 10μ diam.)

Poikilocytosis:

Variation in shape.

Punctate basophilia (basophilic stippling):

Small basophilic particles in the stained erythrocyte.

Howell-Jolly Bodies:

Coarse, round, basophilic particles in the stained erythrocyte.

Cabot's rings:

Thread-like rings, convolutions and figures of eight in the stained erythrocyte.

Hypochromia:

Decreased hemoglobin content of erythrocytes results in a pale stain with widening of the central pallid area.

Polychromasia (basophilia):

A bluish tinge is present in the stained erythrocyte.

Sickle cells (drepanocytes):

Severely malformed erythrocytes, having a sickle, or other bizarre shape.

Ovalocytosis (elliptocytosis):

Erythrocytes are oval or elliptical instead of biconcave.

Target cells (leptocytes)

Thinner (flatter) than normal erythrocytes with central and peripheral areas separated by a paler staining region. Characteristically seen in thalassemia, hemoglobin C disease and severe hypochromic anemias.

Microspherocytes:

Small, round, dark-red staining erythrocytes without central pallor; they are not biconcave. Characteristically seen in congenital hemolytic anemia.

Anemia:

A reduction below normal in the number of erythrocytes, or quantity of hemoglobin, or both.

The Reticulated Red Blood Cell Count

Young red blood cells at the stage when they have just lost their nuclei contain a fine reticulum which stains with "vital stains" in wet preparations. The number found reflects the rate of erythrocyte release by the bone marrow. Reticulocytes are few or absent in hypoplastic anemias and aplastic anemia. They are increased in the hemolytic enemias, such as congenital hemolytic anemia, sickle cell anemia, hemolytic disease of newborn (erythroblastosis fetalis). The deficiency anemias responding to specific therapy show a transient initial increase, followed by a return to normal levels.

Two technics for counting reticulocytes are described below:

Cresyl Blue Method

1. Place two drops of 1 per cent brilliant cresyl blue in absolute ethyl alcohol on a clean concave slide. Let evaporate to dryness (this will take about one minute).

2. After performing venipuncture, place one drop of blood on the dye. Mix with a capillary tube allowing a minimum of 2 minutes for this process.

3. Using the capillary tube to transfer blood-dye mixture to clean glass slides, make thin, even smears.

4. Let air dry and examine with oil immersion lens (if desired, counter-stain with Wright's). Count a total of 1,000 red cells from several portions of the slide and note the number of reticulocytes. The count is divided by 10 and expressed in per cent. The normal count is 5-15 reticulocytes per 1,000 red cells or 0.5 - 1.5 per cent.

New Methylene Blue Method

1. Equal amounts (2 drops) of staining solution and fresh or oxalated blood are mixed in a suitable tube and allowed to stand a minimum of 10 minutes.

2. Mix again before smearing.

3. Thin smears are then made and air dried.

4. Reticulocytes are counted under oil immersion without fixation or counter-staining, as in the cresyl blue method.

5. With this stain the red blood cells are light greenish blue; the reticulum is sharply outlined and deep blue.

Reagents

New methylene blue (color index 927)	0.5 gms.
Potassium oxalate	1.6 gms.
Distilled water .....100.0 cc.	
Filter before use	

Note: New methylene blue, color index 927 must be ordered as such, since this stain is chemically different from the regular methylene blue, color index 922, which is considered unsatisfactory for staining reticulocytes.

Determination of Red Cell Shape

Determination of Microspherocytes in Congenital Hemolytic Anemia

This condition is characterized by the presence of certain abnormal erythrocytes in the blood, namely microspherocytes. These cells are small and more nearly spherical than the normal biconcave erythrocyte. In stained smears they appear uniformly pink, or even darker in the central portion since they are not biconcave.

Sickle Cells

These are red cells which contain the abnormal hemoglobin S. They are most commonly found in members of the Negro race, but they also have been reported in members of the caucasian race. The sickling phenomenon is brought about in vitro by the deoxygenation of the erythrocytes. The action is reversible, the cells returning to a biconcave disc when oxygen is resupplied.

Two methods which demonstrate sickling are:

1. Sodium metabisulfite method - preferred method.

   a. Prepare a 2 per cent solution of sodium metabisulfite by dissolving 200 mg. in 10 ml. of water. This solution kept at 3° or 4° C. will remain effective one week.

   b. Add 2 volumes of above solution to 1 volume of blood in a small tube. Mix, then place 1 drop on a glass slide and seal a coverglass over the preparation with vaseline. Excess blood may be expressed by gently pressing the coverglass with filter paper. Check preparation for sickling after 30 to 60 minutes.

   c. Study the wet portion of the smear. False impressions are obtained from crenation of erythrocytes in the dry portion.

2. Alternate method.

   a. Rim a coverglass with vaseline.

   b. Place a drop of blood on a slide which has been covered by a dried film of 1 per cent solution of brilliant cresyl blue in 95 per cent alcohol.

   c. Place coverglass over drop of blood and press until a thin film is obtained.

   d. Let stand from 2 to 24 hours at room temperature and observe for sickling.

Fragility of Erythrocytes

In certain diseases, notably congenital hemolytic anemia, the fragility of the erythrocytes is markedly increased, as demonstrated by a decreased resistance to hypotonic salt solution. In other diseases such as obstructive jaundice, thalassemia, sickle cell anemia, and chronic hypochromic anemia, the fragility of the red corpuscles is decreased (increased resistance); therefore, this test is a valuable one in the differentiation of several diseases. Reticulocytes have the same fragility as erythrocytes.

The technic for determining fragility of red blood cells is as follows:

1. An accurate 1.0 per cent solution of sodium chloride is prepared using anydrous NaCl (grade C.P.)

2. Two series of 13 tubes, each 10 by 75 mm., are numbered as follows: 68, 64, 60, 56, 52, 48, 44, 40, 36, 32, 28, 24, 20. Place in two racks, one marked CONTROL and one marked PATIENT.

3. Using a 1 ml. serological pipet, add to tube marked 68, 0.68 ml. of salt solution, to tube 64, 0.64 ml. of salt solution; the remaining tubes receive the corresponding hundredths of a ml. of salt solution, as indicated by their markings.

4. Using a 1 ml. serological pipet bring up the volume in each tube to 1 ml. by adding 0.32 ml. of distilled water to tube marker 68, 0.36 to tube 64, etc.

5. Shake briskly to insure thorough mixing of the solutions. Add 1 drop of the whole blood to be tested to each tube. Stopper with clean, salt-free rubber stoppers, and mix well. Return to racks. Let stand at room temp.

6. Read at the end of 2 hours or place in refrigerator overnight and read the next day. The latter gives a more clear-cut reading.

The first tube showing a tinge of reddish-brown in the supernatant fluid is the point of beginning of hemolysis.

The first tube showing a clear red liquid with no macroscopic red cells visible should be considered as complete hemolysis.

The mark on the tubes is the percentage in 0.00 (hundredths) of the salt solution.

With proper salt solution and good technic, the normal control can be kept in the range of hemolysis beginning 0.42-0.44 and hemolysis is complete at 0.32-0.28 per cent NaCl.

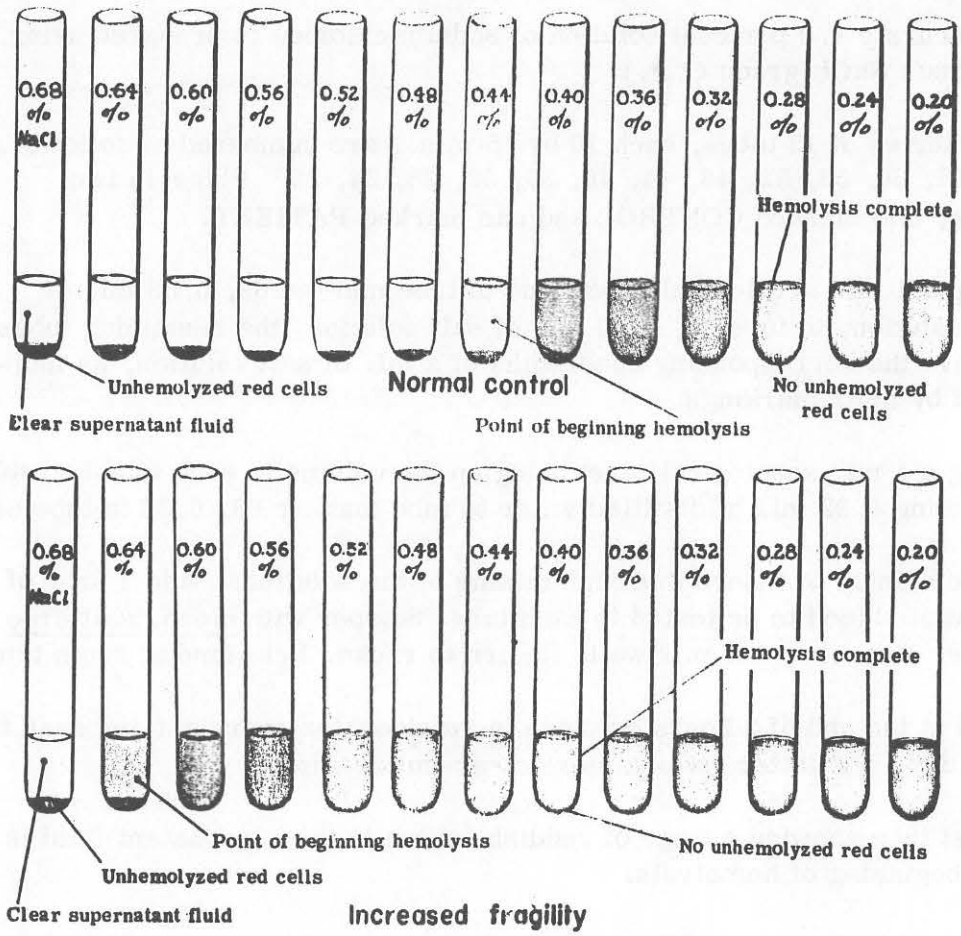
The control should always be reported along with the patient's results, for example:

Patient - Hemolysis begins in 0.64 per cent NaCl.
Hemolysis complete 0.40 per cent NaCl.

Control - Hemolysis begins in 0.44 per cent NaCl.
Hemolysis complete 0.28 per cent NaCl.

Cf. illustration on page 46.

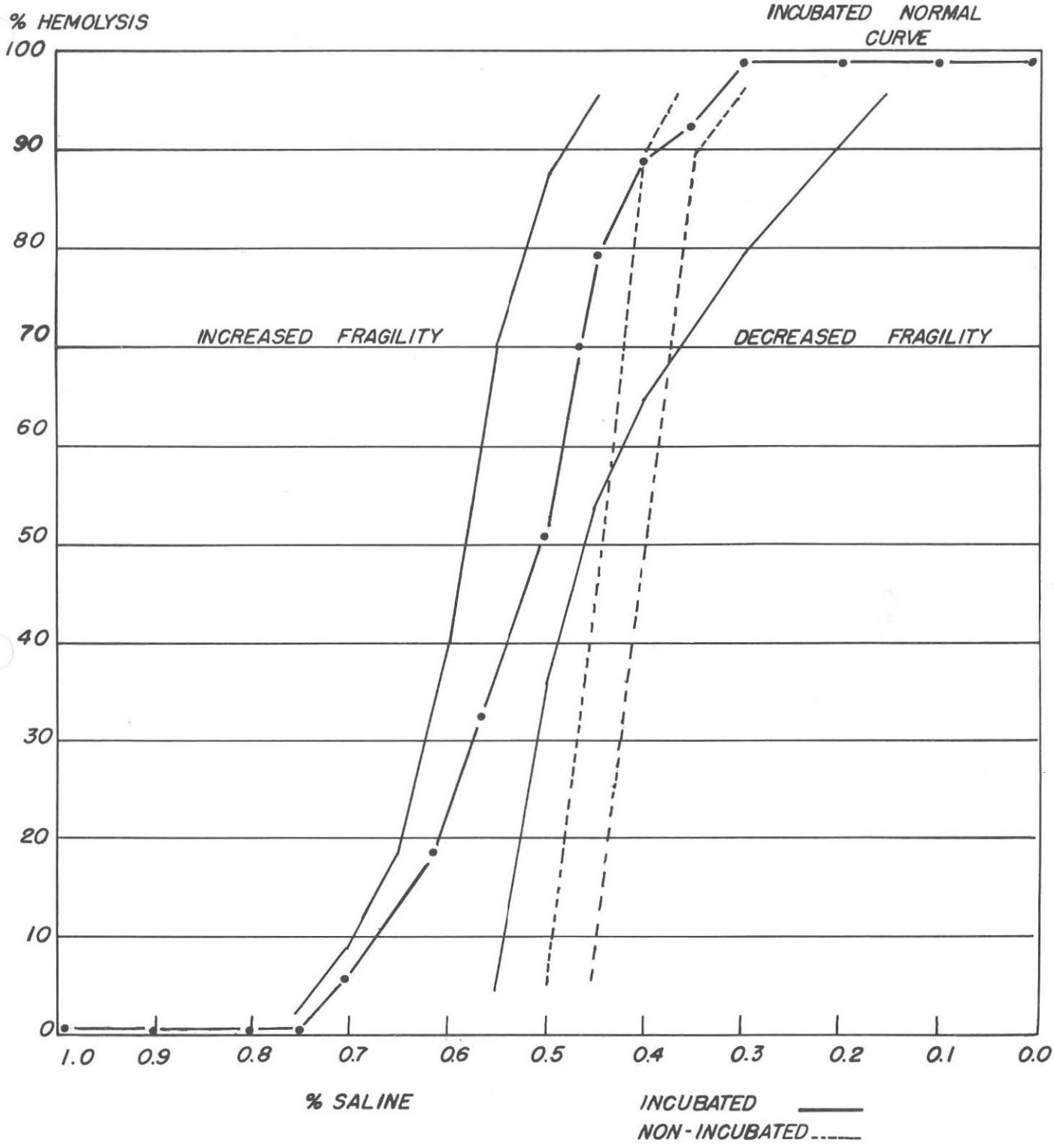
FRAGILITY TEST



Occasionally morphology of the red cells, history, and physical findings are highly suggestive of congenital hemolytic anemia, but the osmotic fragility test is normal. If this occurs a supplemental test may be run which will show increased fragility if microspherocytes or the tendency to spherocytosis are present. The mean cell fragility is increased by 0.15 – 0.2 per cent NaCl while the control increases only about one-tenth as much. The test is called the incubated osmotic fragility test.

Procedure:

1. Collect 1 ml. of oxalated blood in sterile tubes from patient and control.
2. Patient's blood and control's blood are incubated for 24 hours at 37°C.
3. After incubation set up fragility test as described above.
4. If spherocytosis is present, the patient's test will show increased fragility in comparison to the control.


% HEMOLYSIS
INCUBATED NORMAL CURVE
100
90
80
70
60
50
40
30
20
10
0
INCREASED FRAGILITY
DECREASED FRAGILITY
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
% SALINE
INCUBATED
NON-INCUBATED

Erythrocyte Sedimentation Rate

This test measures the amount of sedimentation of the erythrocytes at fixed time intervals, and the total amount of sedimentation during a fixed time, usually 1 hour.

Many different factors influence the sedimentation rate. The red cells carry a negative electric charge and any condition which increases the positive charge in the plasma tends to cause an abnormal aggregation of cells and hence an increased sedimentation rate. Increase in the blood fibrinogen and cholesterol and, to a lesser extent, globulin, have an accelerating effect on the sedimentation rate, while an increase in albumin, nucleoprotein or lecithin have a retarding effect. The presence of bile products, the lipoid equilibrium and nitrogen balance all seem to have an effect. The sedimentation rate is thus a measurement of the balance between a number of constituents in the blood, which either accelerate or retard it. Although non-diagnostic by itself, it does reflect the state of activity of many disease processes and is primarily used for this purpose.

Cutler Method (see illustration)

Cutler divides the sedimentation phenomenon into three phases: (1) the early brief period of rouleaux formation, the size of the rouleaux being the major factor in the rate of sedimentation; (2) a period of true or maximum sedimentation rate; and (3) a period of slower sedimentation caused by the packing of the cells. The most significant figure is the maximum sedimentation rate (MSR), which is the maximum fall within any 5 minute period. This always occurs within the first 30 minutes. Rouleaux formation and hence the sedimentation rate, is due to factors in the plasma. The most significant readings are obtained during the second phase. No correction for anemia need by made in Cutler's method.

The 5 ml. Cutler tube is recommended.

1. Fill the Cutler tube up to the first mark on the scale with 3.8 per cent sodium citrate solution (0.5 ml.).
2. Fill the tube to the 0 mark with whole blood (4.5 ml.).
3. Insert rubber stopper in the tube and invert, mixing thoroughly (carry out the remaining procedure within 3 hours).
4. Place the tube in a vertical position and record the fall in millimeters after 60 minutes.
5. If the fall is 15 mm. or less, draw a straight line graph from 0 to the total fall in 60 minutes. (See illustration on page 48.)
6. Steps 4 and 5 can be eliminated and only step 7 performed if preferred.*

*Since reading the sed rate at 5 minute intervals is tedious, the preliminary 60-minute reading will show which specimens are beyond the normal limits, and only these need be repeated for determination of the MSR.

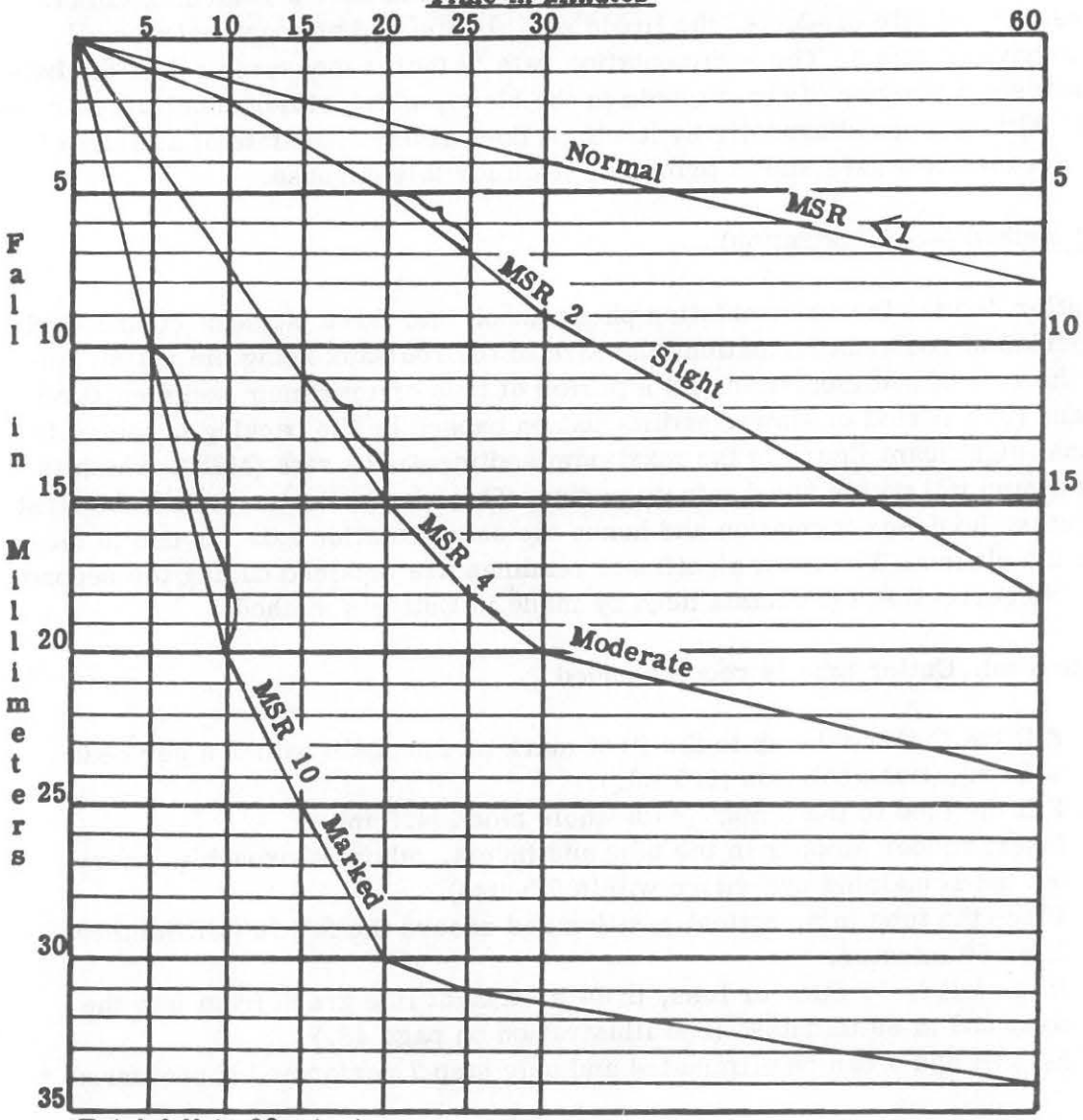
7. If the fall is greater than 15 mm. in 60 minutes, resuspend the cells by gentle inversions. Read the fall in millimeters every 5 minutes for 30 minutes, and again after 60 minutes. Plot the results in graph form (see this page.)

Normal total drop in 60 minutes: male, 0-8 mm.; female, 0-12 mm.

Maximum sedimentation rate (MSR) in any 5-minute period

0-3 mm.	Normal
1-3 mm.	Disease with slight activity
3-8 mm.	Disease with moderate activity
8 plus mm.	Disease with marked activity

BLOOD SEDIMENTATION RATE (CUTLER)



Total fall in 60 minutes_____mm.

Maximum fall in 5 minutes_____mm.

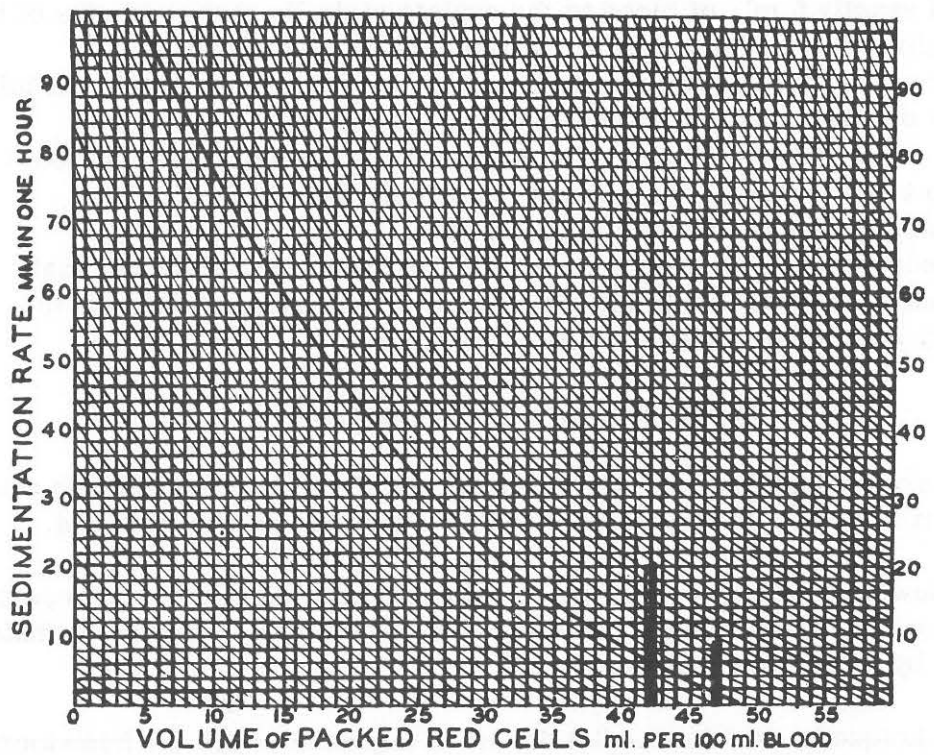
Wintrobe Method

The test is performed on oxalated blood which must be free from any clots or small aggregates of cells. It is important that the test be set up within 3 hours after collection of the blood, and that the blood be at room temperature. The tube should stand vertically. (This is of utmost importance in the long narrow-bore tubes such as Wintrobe and Westergren).

Oxalated blood (see page 3) is thoroughly mixed by gentle inversion, and the Wintrobe tube is filled to the top 0 mark, as for the hematocrit. Stand the tube in a vertical position and record the fall after 1 hour. The tube may then be centrifuged, and the volume of packed cells determined.

Normals: Male 0-9 mm. in 1 hour
Female 0-20 mm. in 1 hour

To correct the Wintrobe sedimentation rate for anemia, use the following chart based on the volume of packed red blood cells.



The horizontal line corresponding to the rate of sedimentation found is followed to the right until it meets the vertical line corresponding to the volume of packed red cells of the patient's blood. The curved line nearest this point of junction is followed downward and to the right until the normal line for men (at 47 ml.) is reached. The corrected sedimentation rate is found by following the horizontal line met at this second junction to the scale on the right.

The open column at 42 ml. and the black column at 47 ml. represent, respectively, the extreme range of uncorrected sedimentation rate found in normal women and men.

HEMATOCRIT DETERMINATION (WINTROBE)

The hematocrit is the volume occupied by the erythrocytes in 100 ml. of whole blood, when packed by centrifugation.

The normal range in males is 47± 5 ml. per 100 ml.; in females, 42± 5 ml. per 100 ml.

1. Place 0.5 ml. of the following anticoagulant solution into a small vial and evaporate to dryness.

   Dry ammonium oxalate ..... 1.2 gms.
   Dry potassium oxalate ..... 0.8 gms.
   Distilled water .....100.0 ml.
   Formalin ..... 1.0 ml.

2. Add exactly 5 ml. of blood to the oxalate vial. No change in size of the erythrocytes is caused by the oxalate in this concentration.
3. Rotate the vial at least twenty times before filling the hematocrit tube.
4. Fill the Wintrobe tube to the 10 mark (right side).
5. Let stand for 10 minutes. This allows the white cells and platelets to rise to the surface, thereby not increasing the cell pack.
6. Centrifuge for 30 minutes at 3,000 rpm.
7. Remove from centrifuge and read the figure on the right hand scale which is on a level with the top of the red cell column. Exclude the buffy coat when reading. Multiply by ten.

Microhematocrit Determinations

There are various high speed centrifuges available at present which allow a hematocrit to be determined rapidly and on a minimal amount of blood.

Free flowing capillary blood from a skin puncture is collected in heparinized precision bore capillary tubing, centrifuged at 25,000 rpm, and the hematocrit determined by a capillary reader.

The technique is useful in pediatrics or in any case where the hematocrit has to be followed closely, as in burn cases. Anesthetists also find a microhematocrit useful in order to follow the condition of a patient during an operative procedure when an ear lobe may be the only available source of blood. Venous blood may also be used when available.

MEAN CORPUSCULAR VOLUME, MEAN CORPUSCULAR HEMOGLOBIN, AND MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION

Discussion:

By using the volume of packed cells (hematocrit), hemoglobin determination, and the red cell count, we can calculate certain mean values in relation to the patient's own blood which are very useful in evaluating hematologic pathology.

Mean Corpuscular Volume, M.C.V.

This is the mean or average volume of a single red cell and is determined by dividing the volume of packed red cells, expressed in milliliters per liter (100 ml.) of blood (the hematocrit x 10), by the number of red cells expressed in millions per cubic millimeter (the RBC count). The result expresses M.C.V. in cubic microns.

Example:

$$\frac{\text{Hematocrit x 10}}{\text{RBC count}} = 82 - 92 \text{ cubic microns (normal)}$$

Mean Corpuscular Hemoglobin, M.C.H.

This is the average weight of hemoglobin contained in a single red cell. It is calculated by dividing the amount of hemoglobin, expressed in grams per 1,000 ml. of blood by the number of red blood cells, expressed in millions per cubic millimeter (the RBC count). The result is expressed in micro-micrograms. (A micro-microgram is the millionth of a millionth part of a gram.)

Example:

$$\frac{\text{grams Hgb x 10}}{\text{RBC count}} = 27 - 31 \text{ micro-micrograms (normal)}$$

Mean Corpuscular Hemoglobin Concentration, M.C.H.C.

This value expresses the average concentration of hemoglobin in the erythrocyte. The amount of hemoglobin, expressed in grams per 100 ml., divided by the volume of packed cells, expressed in milliliters per 100 ml. of the blood (the hematocrit), is multiplied by 100 to obtain the per centile.

Example:

$$\frac{\text{grams Hgb}}{\text{Hematocrit}} \text{ x } 100 = 32 - 36 \text{ per cent (normal)}$$

Although both the MCV and the MCH may have high or low values, the

MCHC never exceeds the normal since the erythrocyte is normally saturated with hemoglobin in relation to its concentration. N.B. The MCV allows us to classify erythrocytes as normal (normocytic), large (macrocytic) or small (microcytic). The MCHC allows us to classify erythrocytes as normal (normochromic) or low (hypochromic); hyperchromia does not occur. MCH is simply a classification by weight.

Formerly both indices and mean values were calculated. The volume, color, and saturation indices correspond to the mean corpuscular volume, mean corpuscular hemoglobin, and the mean corpuscular hemoglobin concentration respectively. Absolute values are preferred to the indices because the absolute values are determined from actual observed figures without reference or reduction to fixed arbitrary normals as is necessary for the calculation of indices.

All of the foregoing figures are important as they are the key to the study of the anemias.

Morphological Classification of Anemia

	Macrocytic	Normocytic	Hypochromic Microcytic
M.C.V.	Increased	Normal	Decreased
M.C.H.	Increased	Normal	Decreased
M.C.H.C.	Normal	Normal	Decreased
Diameter	Greater than 9.0	6.0 - 9.0	Less than 6.0
Cause	Lack of intrinsic or extrinsic factors, or failure to utilize.	Acute hemorrhage Destruction of blood. Lack of blood formation.	Chronic blood loss, deficient supply, or utilization of iron.

MISCELLANEOUS TESTS

Method for Staining Basophilic Aggregations

Principle

Normally 0.5 to 1.5 per cent of erythrocytes show basophilic aggregation. In lead poisoning the percentage of erythrocytes that show basophilic aggregation is greater than 1.5 per cent, and therefore is used as a diagnostic indication of lead poisoning.

Procedure

1. Dry the blood smear from 1 to 3 hours, but not longer.

2. Cover one-half of the smear longitudinally with a strip of filter paper. With a pipet or dropper, apply the minimum amount of methyl alcohol required to moisten the filter paper so that it clings to the slide.

3. Leave until dry and then remove the paper. The methyl alcohol fixes the blood on this portion of the slide. Cover the slide with diluted MANSON'S methylene blue stain and leave for 10 minutes.

4. Wash slide thoroughly 3 to 4 times with distilled water. Dry in air.

5. In the unfixed portion of the slide, count the erythrocytes containing basophilic aggregations in 10 consecutive fields in a row, making a total of 20 fields.

6. In the fixed portion of the slide, count all the erythrocytes in 5 consecutive fields and multiply by 4. (The areas counted in both the fixed and unfixed portions of the slide should be directly adjacent to each other).

7. The outline of the cells in the unfixed portion of the slide are mere shadows and the basophilic aggregations show up as masses, strands, or reticulum taking a blue stain. In the fixed portion of the slide, the erythrocytes are a uniformly clear brilliant blue.

8. The basophilic aggregations count is expressed as a percentage of erythrocytes.

9. $\frac{\text{Erythrocytes with aggregates per 20 HPF}}{\text{Erythrocytes per 5 HPF x 4}} \times 100$ % of erythrocytes containing aggregations.

Reagents

Stock Manson's Stain

Borax (sodium borate)	2.5 gm.
Boiling distilled water	50.0 cc.
Methylene blue	1.0 gm.

Dissolve the sodium borate in the boiling water; add the methylene blue and filter. Dilute before using.

Diluted Manson's Stain

Add 1 cc. of freshly filtered stock stain to 20 cc. of a 0.3 per cent solution of NaCl. This stain must be used the day it is prepared.

Total Circulating Eosinophil Count

Procedure

1. Fill two WBC pipets to the 0.5 mark with blood.

2. Fill to the 11 mark with the special eosinophil reagent.
3. Shake by hand for 30 seconds. Allow to stand for 30 minutes to permit absorption of the stain by the eosinophils. Then shake mechanically for 5 minutes.
4. Discard about 5 drops from each pipet. Charge one side of a hemocytometer with 1 pipet and the other side of the hemocytometer with the other pipet. Let stand 3 minutes.
5. Count all of the cells in the nine 1 square-millimeter areas on each side of the chamber and average these two figures to obtain the mean chamber count.

Note: only eosinophils will be seen in this preparation.

6. Multiply the mean chamber count by 22.2 to obtain the number of eosinophils per cubic millimeter. Results are reported in round numbers only.
7. Normal value: 25 to 300 eosinophils per cubic millimeter.

Example:

Nine squares on one side has a count of eight; nine squares on the other side has a count of ten.

$8 + 10 = 18 \div 2 = 9$ (mean chamber count)

$22.2 \times 9 = 199.8$ or 200 eosinophils per cu. mm.

The factor 22.2 is obtained as follows:

$$\frac{\text{area} \times \text{depth} \times \text{dilution}}{\text{number of squares counted}} = \text{multiplication factor}$$

$$\frac{1 \times 10 \times 20}{9} = 22.2$$

Eosinophil Count Reagent

Mix the following:

50 ml. propylene glycol
40 ml. distilled water
10 ml. 1% phloxine in water
1 ml. 10% sodium carbonate in water.

After filtering, the solution remains stable and usable for at least one month.

LUPUS ERYTHEMATOSIS CELL

Preparation of Substrates:

1. Substrates of normal leukocytes are prepared by placing a rubber ring with an approximate internal opening of 0.5 cm. and height of 0.2 cm. on the center of a clean glass slide. These rings can be simply prepared by cutting them from a rubber tube as used for Bunsen burners or preferably rubber washers of the same dimensions. The area of the slide inclosed by the ring is filled with one to two drops of finger blood or freshly drawn venous blood from a non-lupus patient. The slide is then gently placed in a petri dish, the bottom of which has been covered by a water-moistened filter paper. The petri dish is covered and kept in a warm spot in the laboratory or, better, incubated at 37°C. The moisture prevents the drying of the clot. After one hour, the slide is removed from the petri dish, held perpendicular to the table top, gently sliding the rubber ring away. The slide then is allowed to dry in this position. The area which had been encircled by the ring contains large numbers of white cells which have crept out of the clot with a variable coating of red cells. These preparations may be used as such but can also be washed with saline or serum.

Prodedure of Test:

1. A coverglass, 22 mm. square and of No. 2 thickness, is gently broken into approximately halves between the fingers. Each half is placed approximately 3 cm. apart on the substrate slide. One drop of finger or ear blood from the suspected lupus patient is affixed to the center of a long, clean coverglass (24 x 50 mm.) by gently touching the glass to the drop of blood. The hanging drop of blood is allowed to come into contact with the ring of normal white cells by resting the ends of the long coverglass on the broken pieces of coverglass. No. 1 thickness coverglasses should not be used because these do not afford sufficient elevation, and the blood will spread over the slide rather than retain the shape of a drop. The preparation is placed into a moist petri dish chamber as above. After incubation for one or two hours, the slide is taken out of the petri dish and the coverglass gently lifted from its supports. After the clot has been removed, the excess serum should be quickly removed by forcefully tapping the slide on the table top. The preparation is quickly dried by forceful waving back and forth. It is then stained by the Wright or, after fixation in methanol, with Giemsa method. The area beneath the clot is examined for "L.E." cells.

The authors state that "ring" substrates of both normal and leukemic blood proved to be effective even when used several weeks after being prepared. This is desirable as this material can be prepared beforehand at one's leisure and be available when "L.E." cell formation in the blood of a suspected patient must be tested. It has been our impression, however, that preparations less than one

week old give the best yield of "L.E." cells, and it is advised that none be used that are over one week old.

By the addition of a simple maneuver, this method can also be employed to test the formation of "L.E." cells by serum of a lupus patient. Two drops of normal fresh blood are mixed with an equal amount of suspect serum placed in a small Widal tube. After coagulation, the clot is decanted onto a clean filter paper to remove the excess serum. Two pieces of a No. 2 coverglass are placed on the substrate slide. With a needle, the clot is then positioned on the substrate slide, covered with a coverglass and incubated as before in a petri dish. When this clot is removed and the excess serum and blood cells forcefully removed by tapping, a thin peripheral rim of white cells will be seen to have crept out of the clot onto the substrate slide. This area should be examined for "L.E." cells.

L.E. Cell Evaluation

The L.E. cell is a neutrophil which has phagocytized nuclear material which forms a homogenous globular mass intracellularly. L.E. cell formation depends on a poorly understood plasma factor. This plasma factor is a gamma globulin which can be destroyed by heating to 65°C., but is stable when frozen or at refrigerator temperature. L.E. cell formation is enhanced when serum rather than plasma is used as a source of the L.E. Factor.

Wright's stained preparations produce a characteristic homogenous purple-red appearance of the intracytoplasmic inclusion of the neutrophil. The leukocyte's nucleus is markedly compressed and distorted into a peripheral ring. A criterion of almost complete homogeneity in the inclusion must be strictly adhered to when identifying L.E. cells.

"Tart" cells may be mistaken for L.E. cells by the unwary. The tart cell is usually a monocyte which has ingested nuclear material (nucleophagocytosis). The ingested nuclear material retains recognizable nuclear clumps, nucleoli, or nuclear membrane fragments; it lacks the homogeneity of the L.E. inclusion.

The formation of L.E. cells alone does not establish the diagnosis of Lupus since other conditions may produce (or at least mimic) them. Nevertheless there is a high degree of positive correlation between true L.E. cells and the disease. ACTH or steroid therapy may abolish L.E. cell formation so that a patient receiving such therapy may change from "positive" to "negative" for L.E. cells.

BLOOD COAGULATION

Under normal conditions the blood clotting mechanism begins the moment the blood leaves the circulatory system. After 2 to 6 minutes the blood loses its fluidity and becomes a jelly-like clot. The jelly-like consistency is due to the presence of fibrin strands which are interlaced throughout the clot holding all the cellular components of the blood within their meshes. Upon further standing, the blood thrombocytes adhere to the fibrin strands and cause them to bend or shorten by means of either a physical or chemical reaction. This is clot retraction with expression of serum.

There are two main theories of blood coagulation, and the main difference is whether Factor V and Factor VII act in phase I or phase II of coagulation. This has unfortunately resulted in confusion. A part of the confusion results from the numerous names given to similar factors concerned with coagulation (Table 8). Attempts are currently underway to standardize the coagulation nomenclature.

The time occupied by the thromboplastin-forming reactions accounts for most of the time needed for blood to clot, and it is occurrence of faults in this system which accounts for most of the hemorrhagic states due to defective coagulation. It is important to remember that all reactions in the three phases occur at the same time once thromboplastin has been formed.

The whole process of coagulation is initiated as soon as thromboplastin becomes available. Thromboplastin is not a component of the blood, and it must be generated. Thromboplastin, which the British state is tissue or incomplete thromboplastin, is present in tissues and tissue juices so that when injury takes place, the thromboplastin in tissue immediately starts the coagulation process locally, but it must be supplemented by generation of thromboplastin within the blood by the thrombocytes and plasma factors. Tissues that have a high content of thromboplastin are (1) brain, (2) lung, (3) placenta, and (4) decidua. Thromboplastin is generated in the blood from substances that exist in the blood. Of first importance are the thrombocytes. As they break up, they yield a substance which has been called thromboplastinogenase or platelet factor which then reacts with a group of blood globulins (plasma factors). These are four in number and consist of antihemophilia globulin (AHG), plasma thromboplastin component (PTC), plasma thromboplastin antecedent (PTA), and Hageman factor (glass promoting factor).

In the second phase of coagulation active thromboplastin, Factor V, Factor VII, prothrombin and calcium react to form a small amount of thrombin. This small amount of thrombin causes more thrombocytes to agglutinate and lyse with more thromboplastin formed. More important is that the thrombin accelerates the formation of more thrombin by assisting in converting the inactive forms of

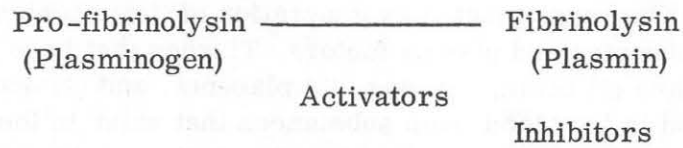
Factor V and Factor VII into more active forms. The calcium is necessary for the action of Factor VII.

In the third phase fibrinogen and thrombin react to form fibrin. This step probably involves the polymerization of fibrinogen to form fibrin.

The occurrence of abnormalities in the first phase account for most of the hemorrhagic states due to defective coagulation. If thrombocytes are deficient, we clinically have a thrombocytopenic purpura with an abnormal clot retraction, prolonged bleeding time, and a normal clotting time. If there is a deficiency of one of the plasma factors, the clotting time is prolonged with a normal bleeding time and prothrombin time.

Abnormalities in the second phase, a deficiency of Factor V, Factor VII, or prothrombin, prolong both the clotting time and prothrombin time, leaving a normal bleeding time.

The abnormality of the third phase is fibrinogen deficiency. Deficiency of fibrinogen is one of the rarest types of coagulation defect and is found in three sets of circumstances. Congenital inability to manufacture fibrinogen in sufficient amounts is the rarest of all the types. Second are those circumstances complicating labor, and almost all of these are cases of abruptio placenta. Third are those circumstances arising as a complication of major surgery, particularly thoracic or retroperitoneal surgery, which may be ascribed to the lysis of fibrinogen by a fibrinolytic system, presently still the subject of investigation. The fibrinolytic system is believed to be responsible for keeping the body's blood vessels free of fibrin deposits. The deposits may spontaneously form as a result of trauma or other less well understood events, e.g., phlebothrombosis. This system may be simply depicted as follows:



Under normal circumstances the active enzyme, fibrinolysin, exists in minute amounts in the blood. Under poorly understood circumstances "activators" may be released in large quantity to convert pro-fibrinolysin to fibrinolysin. If fibrinolysis occurs as a sequel, plasma coagulation factors, especially fibrinogen, are affected.

Fibrinolytic activity is apparently decreased following large doses of ionizing radiation. This situation is currently under study.

All laboratory techniques used to detect defects of the clotting mechanisms are based on observations on fibrin formation and the certainty of information obtained decreases with separation from this stage. Table 9 contains biochemical characteristics which aid in differentiation of the various clotting factors.

Figure I

MODERN THEORY OF COAGULATION

Phase One - Thromboplastin Generation (2-5 minutes)

(a) Thrombocytes + Plasma Factors (AHG, PTA, PTC, Hageman) ⟶ Thromboplasin

(b) Tissue injury ⟶ tissue thromboplastin

Phase Two - Thrombin Formation (Prothrombin Conversion) (10-15 seconds)

Thromboplastin + Prothrombin + Factor V + Factor VII + (Factor X?) $\xrightarrow{Ca}$

Phase Three - Fibrin Formation (10-15 seconds)

Thrombin + Fibrinogen ⟶ Fibrin

Recommendations for Standardization of coagulation terminology are presented in the article by Wright (Cf. bibliography).

TABLE 8

TERMINOLOGY

Factor

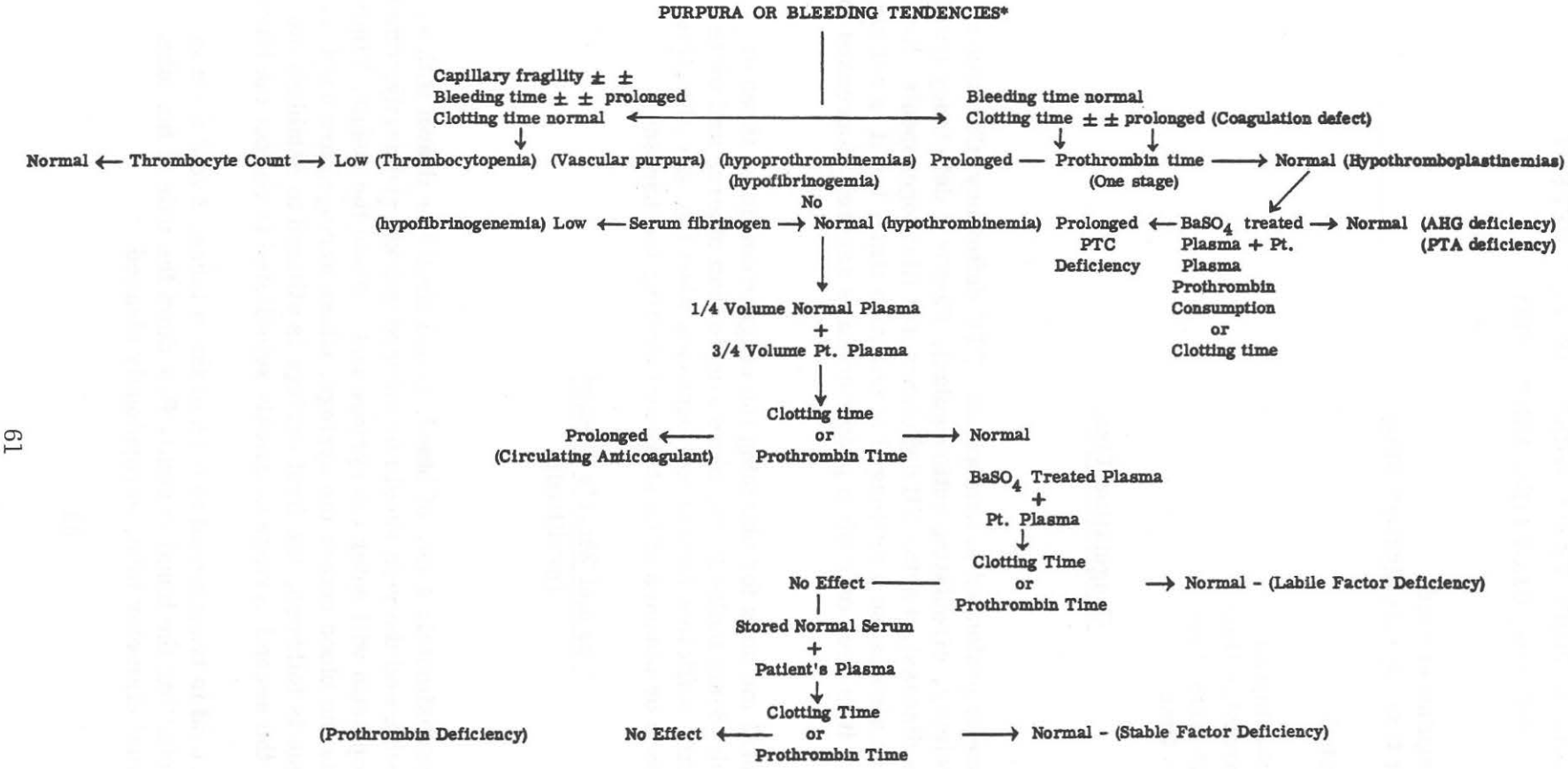
AHG	- antihemophiliac globulin factor, factor VIII
PTC	- plasma thromboplastic component factor, Christmas factor, factor IX
PTA	- plasma thromboplastic antecedent factor
Factor V	- labile factor, accelerator factor, plasma Ac-globulin, proaccelerin
Factor VII	- stable factor, serum prothrombin conversion accelerator (SPCA), proconvertin, prothrombin accelerator
Hageman factor	- glass promoting factor
Factor X	- Stuart Prower Factor

TABLE 9

BIOCHEMICAL CHARACTERISTICS WHICH FACILITATE DIFFERENTIATION BETWEEN THE VARIOUS CLOTTING FACTORS

	AHG	PTC	PTA	Prothrombin	Factor VII	Factor V	Fibrinogen
Type protein (globulin)	+	+	+	+	+	+	-
Presence in serum	-	+	+	-	+	-	-
Utilization during clotting	+	-	±	+	-	+	+
Presence in stored plasma	-	+	+	+	+	-	+
Adsorption from oxalated plasma by $BaSO_4$, $Al(OH)_3$	-	+	-	+	+	-	-
Adsorption from serum by $BaSO_4$	-	-	+	-	+	-	-

TABLE 10



* Levinson, S. A. & MacFate, R. P., 1956. Clinical Laboratory Diagnosis. 5th ed., Philadelphia, Lea & Febiger

ROUTINE LABORATORY TESTS WHICH DETECT DEFECTS OF THE CLOTTING MECHANISM

1. Whole blood coagulation time
2. Plasma clotting time (recalcification time)
3. Bleeding time
4. Thrombocyte count
5. Clot retraction
6. Prothrombin consumption
7. One stage prothrombin time
8. Whole blood clot lysis time
9. Euglobulin lysis time

Coagulation Time

Discussion:

Coagulation time is prolonged in hemophilia, PTC deficiency (Christmas disease), PTA deficiency, circulating anticoagulant, Factor V deficiency (parahemophilia, Owen's disease), Factor VII deficiency and fibrinogenopenia. Hypoprothrombinemia can also cause a prolonged coagulation time, but it is not significant clinically as it is seen only when prothrombin is severely depressed to 5 per cent or less.

There are several methods for obtaining the coagulation time, three of which are described: Some methods are more simple than others, and while less accurate are still sufficient for clinical purposes when the only objective is to note the presence or absence of an abnormal clotting mechanism.

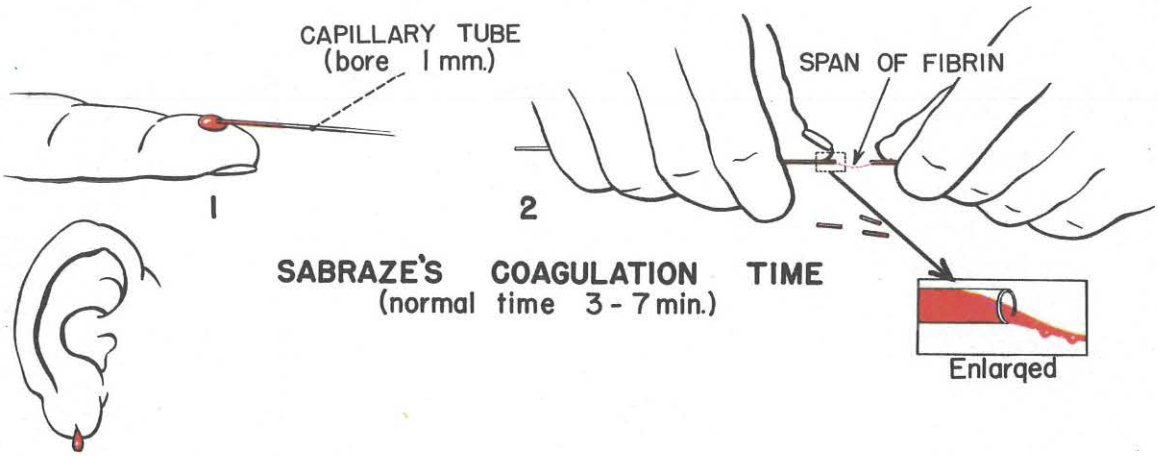
Lee and White's Method (modified)

Procedure

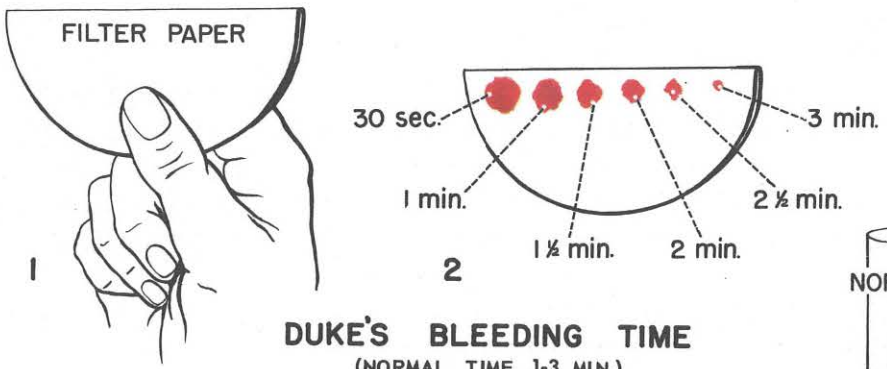
1. Withdraw approximately 4 ml. of blood. Blood should be drawn with as little stasis as possible and the vein should be entered quickly and neatly, otherwise tissue thromboplastin will enter the syringe and confuse the result. Timing is started as soon as the blood enters the syringe. Glass syringes are used. A two syringe technique is followed, the first syringe is utilized to establish the venipuncture, then the second syringe is quickly substituted to collect the blood.

2. One cc. of blood is transferred to each of three tubes. Avoid foam in the specimen by permitting the blood to gently flow down the side of the tube. Use a 12 mm. internal diameter tube, scrupulously cleaned.

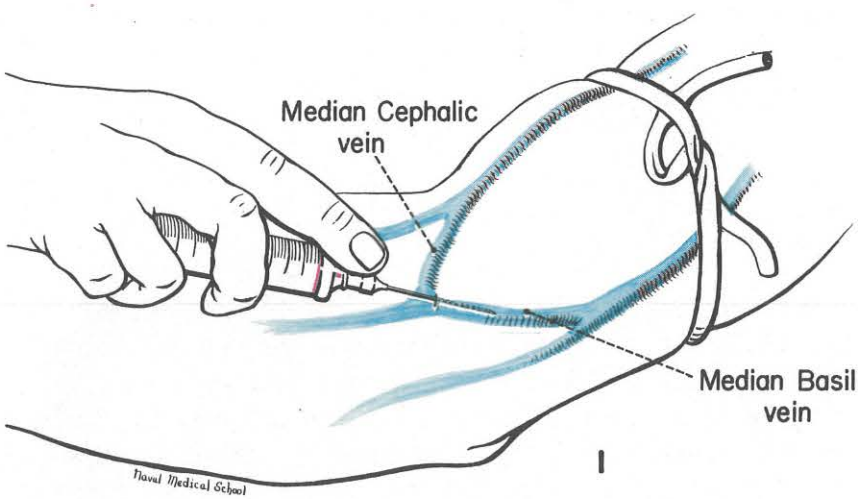
TESTS OF THE CLOTTING MECHANISM



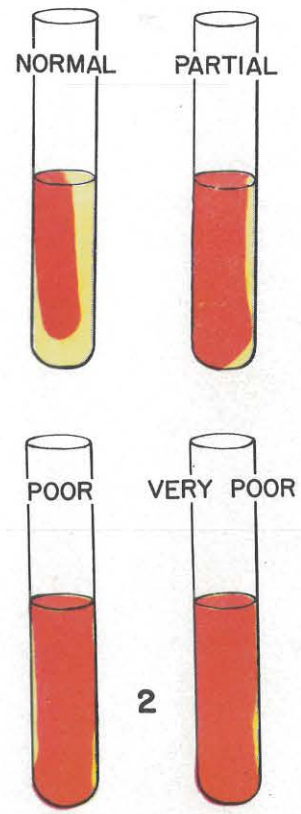
SABRAZE'S COAGULATION TIME
(normal time 3 - 7 min.)



DUKE'S BLEEDING TIME
(NORMAL TIME 1-3 MIN.)



CLOT RETRACTION



3. Set the tubes in a rack and after 3 minutes the first tube is inclined to observe the state of fluidity. This observation is repeated every 30 seconds. The end point is the moment the clot is firm enough to permit holding the tube in a horizontal position without flow of blood.

4. Pick up the second tube at this point, and observe at 30 second intervals until clotting occurs. Examine the third tube in like manner. The coagulation time is calculated from the time the blood enters the syringe until the clot is firm in the third tube.

Normal: 6-16 minutes with a tube of internal diameter of 12 mm.

Note: The clotting time increases as the internal diameter of the tube increases.

Interpretation

A clotting time of 16 to 20 minutes is suggestive of hemorrhagic tendency; if it is prolonged beyond 20 minutes it is indicative of hemorrhagic disease.

Silicone Tube Method

Discussion

To siliconize tubes or syringes, the procedure must be done in the open air or at a window as the white fume given off is hydrochloric acid. Caution: Silicone should never be aspirated by mouth and every precaution should be made to keep it away from the skin as well as the lungs. All tubes used in the procedure given below should first be thoroughly cleaned and rinsed well with saline. Siliconized tubes should be kept separate. If necessary, known silicone can be removed by soaking tube in absolute ethyl alcohol saturated with potassium hydroxide sticks. Do not attempt to siliconize rubber tubing or needles. One technician should do all the siliconing, and also be responsible for the test itself.

Procedure

1. Dilute silicone (ex. Clay-Admas "Siliclad) 1:100 with water.
2. Immerse objects to be coated for 5 seconds or longer. Be sure that all surfaces inside the tube are covered with the solution.
3. Rinse with distilled water 10 times.
4. Dry at 100°C. for ten minutes, or at room temperature for 24 hours. Syringes have to be dried with the plunger removed from the barrel or the syringe will be jammed.
5. Cool, and the tubes are ready for use.

Test - Use a two syringe technique

1. Use siliconized syringes to draw blood, i.e., syringe No. 2 is siliconized.
2. Gently expel blood with the needle removed from the syringe, into tubes until one-fourth to one-third full.
3. Place in rack and do not touch for 15 minutes. Tilt tube very gently towards horizontal position about every 5 minutes hereafter. Record time when tube can be tilted to horizontal position without flow of blood.

Normal clotting time by this method is 25-35 minutes.

Sabraze's Method

Discussion

A capillary tube 8 cm. in length by 0.8 to 1.2 mm. in diameter is used. Holding the tube in the palm of the hand to approximate body temperature will give more accurate results. This method may fail to show prolonged coagulation time due to admixture of tissue juices, and is the least desirable of all methods. It is imperative that free flowing drops be obtained and that the finger is not squeezed to obtain blood.

Procedure

1. Cleanse the finger with alcohol and allow to dry.
2. Puncture finger, the first 2 drops are gently wiped away.
3. Note the time at the appearance of the third drop.
4. Allow the tube to fill by capillarity.
5. At the end of 2 minutes break off a small portion of the tube and carefully separate the broken ends.
6. Repeat this maneuver every 30 seconds after the first 2 minutes until a thread of fibrin spans the space between the broken ends of the tube as they are separated.
7. The coagulation time will be the time which has elapsed between the appearance of the third drop on the finger and formation of the fibrin strand between the edges of the broken capillary tube.

Note: The normal coagulation time by this method is 3 to 7 minutes.

Plasma Clotting Time (Recalcification Time)

Discussion

This is a nonspecific type of test, similar to the whole blood coagulation time, in which the coagulation time of plasma is measured. Its greatest value is in the

diagnosis of hemophilia. It is prolonged in hemophilia, afbrinogenemia, circulating anticoagulant, deficiencies of PTC, PTA, Factor V, Factor VII, and prothrombin. It is also prolonged in thrombocytopenias in contradistinction to the coagulation of whole blood which is normal.

Procedure

1. Mix 4.5 ml. of venous blood with 0.5 cc. of 0.1 M sodium oxalate.
2. Centrifuge blood at 1,000 rpm and remove plasma.
3. Incubate 0.1 ml. of plasma in a 37.5°C. water bath for 3 minutes.
4. Start to time while adding 0.2 ml. of 0.01 M calcium chloride and mix.
5. Check at the end of 60 seconds and every 15 seconds thereafter until plasma is completely coagulated.

Normal: 90-120 seconds.

Reagents:

0.01 M Calcium Chloride: Dissolve 1.1 grams of anhydrous calcium chloride and 4.2 grams of anhydrous sodium chloride in distilled water and q. s. to 1000 ml.

0.1 M Sodium Oxalate: Dissolve 13.4 grams sodium oxalate, reagent grade, anhydrous, in distilled water and q. s. to 1000 ml.

Bleeding Time

Discussion:

The bleeding time is the time required for a small cut to cease bleeding. The role of the thrombocytes is so vital (see thrombocytes) that a prolonged bleeding time may result if the thrombocytes are greatly reduced.

Duke's Method

1. Cleanse the finger or lobe of the ear (preferably the ear) with alcohol and allow to dry.
2. Make a puncture with a hemolet. Note the time blood appears.
3. At half minute intervals blot with a piece of absorbent paper (do not touch the skin) until bleeding has ceased.

Normal: 1-3 minutes. In some pathological conditions, bleeding may continue for several hours. When at the end of 20 minutes the size of the drop is the same as the first one, it is a good policy to stop the bleeding and make this report: "Bleeding time incomplete in 20 minutes."

Ivy's Method

1. Apply sphygmomanometer cuff around the arm and apply 40 mm. pressure.
2. Puncture pronator surface of forearm with a Bard-Parker #11 blade attempting to make a wound of 2 mm. depth and length. Absorb blood by filter paper every 30 seconds with Whatman #1 filter paper as in Duke's method.

Normal: Normal bleeding time for this method is 1 to 6 minutes.

THROMBOCYTES (Platelets)

Discussion:

The chief function of the blood thrombocytes appears to be their control of the process of coagulation. Thrombocytes are essential for the formation of thromboplastin of the plasma, and they bring about retraction of the clot. They are important in protecting the walls of blood vessels when the endothelium becomes injured, since they adhere to the injured surfaces. They collect around cuts, traumatized surfaces and foreign bodies. When a blood vessel wall is injured or perforated the thrombocytes immediately accumulate in the area of injury. Adhering to one another, and to the edges of the injured areas they seal over the involved vessel wall. At the same time, an intensely active vasoconstrictor substance (serotonin) from the thrombocytes is released which acts on the injured vessel so as to maintain its initial contraction. It also causes contraction of other vessels in the vicinity. Serotonin acts only on vessels with muscular walls and not on the capillaries.

The number of thrombocytes in the human blood is difficult to determine because of a tendency to disintegrate, and because they adhere to foreign bodies and to each other. Most methods of counting have normal ranges from 140,000 to 700,000 per cu. mm. If a patient's thrombocytes are reduced below 60,000 per cu. mm., a hemorrhagic tendency usually becomes clinically evident. Thrombocytes are irregular in size and shape, average 2-4 microns in diameter, contain fine azurophil granules, and stain pale lilac with Wright's stain. The normal life of a thrombocyte is about 5-7 days.

Thrombocyte counts are usually increased in tuberculosis, secondary anemia, and chronic granulocytic leukemia. Increased thrombocytes are seldom of clinical significance of themselves.

Reduction in thrombocytes (thrombocytopenia) is found accompanying infectious disease, pernicious anemia, lesions involving the bone marrow, the acute leukemias, and in acquired (secondary) and idiopathic thrombocytopenias. An important differential diagnostic point in thrombocytopenia and hemophilia is a normal thrombocyte count in the latter. Removal of the spleen in certain thrombocytopenic states is followed by a rise in thrombocytes which results in clinical improvement.

It has been recently shown that thrombocytes have agglutinogens. The plasma normally contains no agglutinins to platelet agglutinogens until sensitization has occurred. This may be one reason why after a thrombocyte transfusion there sometimes is no rise in the circulating thrombocyte count. An even more important factor is the physical state of the platelets infused. If they are grossly clumped when infused, they quickly disappear from the circulation.

Two syndromes in the past have been attributed to functional abnormalities of the thrombocytes; von Willebrand's disease and thrombasthenia. It is now accepted that in von Willebrand's disease the thrombocytes are normal and that it is due to an abnormality of the capillaries.

THROMBOCYTE COUNT

There are two methods: (1) direct, and (2) indirect.

Thrombocyte counting with the phase microscope is the only accurate method and is preferred if the equipment is available. However, other direct and indirect methods are described since they are satisfactory for screening purposes.

Direct Method

I. Phase Method

Equipment

2 red blood cell pipets
1 phase hemocytometer (flat bottom - Improved Neubauer ruling)
1 phase microscope with 15x oculars
1% ammonium oxalate to which 5 cc. of 10% formalin is added to 1000 cc. as a preservative
Thin coverslips (22 mm. - No. 1-1/2)

Procedure

1. A tube of oxalated blood from venous puncture is preferred. If no puncture is advisable or no other procedure is requested requiring a tube of oxalated blood, then a finger puncture may be made. A free flowing drop of blood is essential. The count should be performed within one hour of collection of the specimen.

2. The blood is drawn to the 1.0 mark in each of two red blood cell pipets, and then diluted to the 101 mark with 1% ammonium oxalate. One pipet is used as a check against the other, so that all counts are run in duplicate.

3. Shake the pipet for at least five minutes. Fill one side of a hemocytometer with one pipet and the other side with the second pipet. Discard the first 5 drops from the pipet before filling the hemocytometer.

4. The charged hemocytometer is placed in a covered petri dish, which has a damp piece of filter paper in the bottom to prevent drying the specimen. Allow to set for 20 minutes to allow the thrombocytes to settle out.

5. Count the thrombocytes (on both sides of the counting chamber) in the same squares (5 small squares) as are routinely counted in doing a RBC.

6. Divide the total number of thrombocytes counted on both sides of the chamber by 2 and multiply by 5,000. This gives the number of thrombocytes per cubic millimeter.

7. When there is a question of even distribution in the counting chamber, or the thrombocyte count is below 125,000/cu. mm., count all 25 squares in the center sq. mm. area. The multiplication factor then used is 1,000.

Thrombocytes viewed by phase appear as lilac colored bodies approximately one-half the size of a red cell. Bacteria may clump or group and give a false impression of being a thrombocyte.

Appearance of Thrombocytes:

Size - 1 to 5 microns
Shape - round, oval or comma-shaped. They may have small processes at their periphery.

Normal:

Normal values for phase thrombocyte counts are slightly lower than that of the Fonio method; the range is 140,000 to 400,000 with this method.

Indirect Method

I. Fonio Method

Procedure

1. Place 1 drop of 14 per cent aqueous solution of magnesium sulfate on the finger.
2. Make puncture through the drop of solution and allow blood to collect on top of the solution.
3. Make regular blood films; dry in air.
4. Stain with Wright's and examine under oil immersion. Count 1,000

cells and record the number of thrombocytes seen.

5. The erythrocyte count divided by 1,000, multiplied by the thrombocytes counted gives the number of thrombocytes per cu. mm. For example:

If the red blood count was 5,000,000 per cu. mm. and 65 thrombocytes were encountered while counting 1,000 red cells:

$$\frac{5,000,000}{1,000} = 5,000 \times 65 = 325,000/\text{cu. mm.}$$

The normal thrombocyte count by this method varies between 200,000 and 400,000/cu. mm. Counts below 100,000 per cu. mm. should be checked carefully. Hemorrhage usually appears in counts below 60,000 per cu. mm.

Do not forget to do a red blood count when using the Fonio method.

CLOT RETRACTION

In certain diseases, notably thrombocytopenic purpura, clot retraction is markedly abnormal due to lack of thrombocytes.

Procedure

1. Collect 5 ml. of blood from a venipuncture and place in a 10 by 75 mm. test tube.
2. Stopper tube. Place in 37°C. water bath.
3. Examine at the end of 2 hours, and again at the end of a 24-hour period, report the results as:
   a. Normal or complete retractility
   b. Partial retractility
   c. Poor retractility
   d. Very poor retractility

Normal: The blood coagulum begins to retract and express serum at the end of 1 hour and is complete at the end of 24 hours or less.

Occasionally the blood adheres to the wall of the tube and does not retract at all. If no retraction has taken place in 2 hours, it is advisable to rim the clot gently with a wooden applicator stick.

THE ANTIHEMOPHILIAC FACTORS (Plasma Factors) (PTA, PTC, and AHG)

The first antihemophiliac plasma factor which was discovered was antihemophiliac globulin (AHG). Until recently, all hemophilia was thought to be due to a deficiency of this factor as normal plasma would correct the clotting defect. In 1952 a case of hemophilia was found, and in the course of the investigation the plasma of a known hemophiliac was mixed with the blood of the patient. The result was correction of the clotting defect. On the basis of this case, it was postulated that two factors were present in normal blood, and one factor was missing from each of the hemophiliacs. Subsequently, still another patient was found whose blood corrected the clotting defect of each of the other two, and a third factor was postulated. The second and third factors were named plasma thromboplastin component (PTC) and plasma thromboplastin antecedent (PTA), respectively. A lack of PTC has been termed "Christmas Disease," after the patient in whom it was first encountered.

Interference with the clotting mechanism in hemophiliacs and hemophilioid diseases is primarily in Step 1, where formation of thromboplastin is defective.

The role of the plasma factors would seem more crucial in the formation of thromboplastin than thrombocytes, since in hemophiliacs and hemophiliod diseases the actual coagulation of the blood is prolonged, whereas it is normal in thrombocytopenia.

In the usual case of thrombocytopenia, sufficient thromboplastin is elaborated for blood clotting to take place within the normal length of time even though the thrombocytes are diminished. The prothrombin consumption is delayed, however, which means that no excess thrombocytes are present. Markedly reduced thrombocytes will also delay the clotting, though it is unusual to find thrombocytopenia of such severity.

True hemophilia presents a deficiency of antihemophiliac globulin of such degree as to cause delayed clotting, as well as defective prothrombin consumption. Normally, blood contains large amounts of all clotting factors, marked deficiencies must be present in order to delay the clotting mechanism.

Fortunately, certain properties of the plasma factors are sufficiently characteristic to be of diagnostic significance. It has been found that if normal human plasma is mixed with insoluble barium sulfate, the barium sulfate will absorb and remove from the plasma the PTC and prothrombin. It has also been found that normal human serum which has been allowed to stand for 48 hours will contain neither AHG nor prothrombin. These properties may be schematically represented as follows:

	Normal $BaSO_4$ treated plasma	Normal 48-hr. old serum
AHG	Present	Absent
PTC	Absent	Present
PTA	Present	Present

It is evident from the scheme that if normal $BaSO_4$ treated plasma and normal 48-hour old serum are added to the blood of persons with a clotting abnormality, the defective component can be determined by whether or not the clotting defects (clotting time and prothrombin consumption time) are corrected. This may be represented as follows:

	Normal $BaSO_4$ treated plasma	Normal 48 hr. old serum
AHG defect	Corrected	Not corrected
PTC defect	Not corrected	Corrected
PTA defect	Corrected	Corrected

Method

A. 1. Collect 4 ml. oxalated blood from patient.
2. Centrifuge and remove plasma
3. Add 0.1 ml. patient's plasma to 0.1 ml. calcium chloride (.04 molar) in 37.5°C. water bath; determine recalcification (clotting) time.

B. 1. Add 0.16 ml. patient's plasma to 0.4 ml. barium sulfate absorbed plasma.
2. Incubate at 37.5°C. for several minutes and then pipet 0.1 ml. of the mixture into another test tube in same water bath.
3. Add 0.1 ml. calcium chloride and note time of clotting.

C. 1. Repeat same steps as under "B" but use 48-hour old serum instead of barium sulfate absorbed plasma.
2. Use preceding chart for interpretation.

Reagents

Barium sulfate absorbed plasma

Add 100 mg. of powdered barium sulfate to each 1.0 ml. of oxalated plasma. Incubate for 10 minutes at 37.5°C.; shake every 3 minutes. Centrifuge at 3,000 rpm for 30 minutes. Keep refrigerated until ready for use, which should be within 2 hours.

PROTHROMBIN CONSUMPTION TEST

In phase 2 of the coagulation scheme (Figure 1), prothrombin is converted to thrombin by the action of thromboplastin. Prothrombin is present in whole blood in excess quantities, so that at the moment the actual clot occurs, unconverted prothrombin remains. The excess prothrombin is converted by thromboplastin as time passes, and measurement of this is called the prothrombin consumption test. If there is any abnormality in thromboplastin formation, the conversion of prothrombin to thrombin will be delayed or absent. The methods used for this assessment depend on comparing the coagulant ability of plasma and serum by the addition of thromboplastin and calcium chloride at a determined time after coagulation has occurred. It is empirical and not a true measure of prothrombin. It is of interest that, in the test tube, only part of the prothrombin is converted to thrombin in coagulation of all the fibrinogen of the blood.

It has been found that in the vast majority of normal individuals less than 20 per cent of the prothrombin present in the plasma remains in the serum one hour after coagulation of the blood has occurred. In hemophilia and thrombocytopenic purpura, prothrombin consumption is much less complete with more than 70 per cent remaining.

Though thromboplastin is normally present in the tissue fluids, it will not be elaborated in the blood if the first step of the clotting mechanism is defective. Thrombocytes are essential to the normal evolution of thromboplastin; hence, reduced thrombocytes cause a delayed prothrombin consumption.

In deficiencies of the antihemophiliac factors, there is also a deficiency of thromboplastin formation due to lack of antihemophiliac globulin, plasma thromboplastin component or plasma thromboplastin antecedent. The prothrombin consumption is poor. In cases of afibrinogenemia, prothrombin consumption is normal.

Prothrombin Consumption Test

Procedure

1. Collect blood from the patient with a clean venipuncture using a 2-syringe technique. Place approximately 2.0 ml. into each of two saline-rinsed 12mm. test tubes.
2. Allow the blood to coagulate at room temperature.
3. Place the two tubes in a 37°C. water bath for 2 hours.
4. Gently free the clot from the sides of the tubes and centrifuge at 1500 rpm for 5 minutes.
5. Collect the serum from the two tubes and pool it in one tube.
6. Incubate the serum at 37°C. for ten minutes to destroy any residual thrombin. (If desired, the serum can now be refrigerated for a maximum interval of 1 hour at 4-6°C. without loss of potency.)
7. Into each of three small serological test tubes in a 37°C. water bath, place 0.1 ml. of prothrombin-free plasma. (Cf. reagents below).
8. Add 0.1 ml. of "Simplastin" solution to each tube.
9. Pipet 0.1 ml. of serum into the first tube; simultaneously start a stop watch. Draw a loop through the solution until gel-strand formation occurs, thus determining the prothrombin time.
10. Repeat the procedure with the second tube. The third tube is the control and should remain fluid, indicating the absence of prothrombin in the prothrombin-free plasma.
11. A control should be run simultaneously using blood from a normal person.

Reagents

1. Prothrombin-free plasma

Pool several of the plasmas on hand from the day's routine plasma prothrombin determinations. Do not use abnormals. To 5 ml. of the pooled plasma add 1.0 ml. of the 30% suspension of barium sulfate, mix and incubate ten minutes at 37°C. Mix once or twice again during the ten minutes. Centrifuge for several minutes and then decant the prothrombin-free plasma into another tube.

2. Barium sulfate suspension, 30 per cent

Suspend 30 gms. of barium sulfate in 100 ml. of distilled water. This is stable indefinitely in the refrigerator.

Note: Commercial fibrinogen products (e.g., Warner-Chilcott) may be used in place of prothrombin-free plasma.

Normal

The normal serum prothrombin time by this method is 25 to 40 seconds which is equivalent to a prothrombin consumption of 91 to 97 per cent. An abnormally short prothrombin time of 12 seconds, such as is found with hemophiliac and thrombocytopenic serums, indicates a virtual absence of prothrombin consumption.

PROTHROMBIN

Prothrombin is utilized in the clotting mechanism in Step 2. A prothrombin defect is reflected in a prolonged one-stage prothrombin time and an abnormal clotting time. The prothrombin consumption curve is normal, since the prothrombin present is adequately consumed within the normal length of time.

Prothrombin is formed primarily in the liver, and its production is dependent upon adequate absorption of vitamin K. Dietary deficiency is never responsible for hypoprothrombinemia, as the chief source of vitamin K is in the gastrointestinal tract where it is synthesized by the intestinal flora. The presence of bile is necessary for the absorption of vitamin K, which is a fat soluble vitamin. It is therefore evident that obstruction of the biliary tract, biliary and gastrocolic fistulae, certain diseases of the liver, and certain disorders affecting the absorption of fat (e.d., sprue) will be accompanied by deficiency of vitamin K. Any of these disorders will result in hypoprothrombinemia.

Hypoprothrombinemia may be induced by the administration of dicoumarol and similar drugs, e.g., Coumadin, Hedulin. It is important to remember that these drugs first lower the level of Factor VII. PTC depression occurs later. The drugs do not directly intervene to delay the clotting of blood, and therefore are not effective anticoagulants in vitro. They act primarily to prevent the formation of prothrombin, Factor VII and PTC in the liver. Administration of dicoumarol does not immediately affect the clotting mechanism as one or two days are required to utilize the previously formed factors. Salicylates produce hypoprothrombinemia by the same mechanism, but are much less powerful, and serious bleeding is rarely encountered as a result of ingestion of salicylates.

Congenital hypoprothrombinemia may also be present. This condition must be kept in mind when dealing with bleeding problems of infancy and childhood.

Prothrombin Time

Principle

This test is a measure of the speed of conversion of prothrombin to thrombin. It is important (1) in evaluating the clotting mechanism of patients on dicoumarol (Coumadin, Hedulin) therapy and (2) in the evaluation of liver disease and hemorrhagic diathesis. This test is the most useful single test of clotting function. The test as originally interpreted was based on one very important assumption - that the speed of prothrombin conversion, in the presence of tissue extract, is uniform in all plasma samples. It is now widely recognized that this assumption is not justified. The speed of prothrombin conversion is affected by two factors, Factor V and Factor VII.

Procedure

1. Add 4.5 ml. of blood to 0.5 ml. of a 0.1 molar solution of sodium oxalate. Mix well. Centrifuge oxalated blood at 1700 rpm for 10 minutes. Pipet clear plasma into a test tube and store in refrigerator. The test should be run within 2 hours.
2. Warm plasma to be tested in a water bath at 37°C. for 5-10 minutes.
3. Pipet 0.2 ml. of "Simplastin" suspension into a test tube and place in water bath at 37°C. for 5 minutes.
4. With 0.1 ml. micropipet, transfer 0.1 ml. of the pre-warmed plasma to the tube containing 0.2 ml. of "Simplastin" suspension.
5. Quickly blow out pipet and start stop watch simultaneously, preferably by a foot treadle. Tap the bottom of tube sharply to mix contents.
6. Immediately insert a small #22 nichrome wire loop stirrer into the tube. Move loop across bottom of tube in sweeping motions about 2 times per second.
7. When clot appears, stop watch and record time. Experience will permit the first formation of clot to be recognized. Always use the same endpoint. Duplicate test should agree to within 1 second.

Reagents

0.1 molar sodium oxalate

Dissolve 13.4 gms. of sodium oxalate, reagent grade, anhydrous, in distilled water and q.s. to 1,000 ml.

Distilled water

pH should not be lower than 6.0. Use freshly distilled water since its pH drops on standing due to CO_2 absorption from room air.

Euglobulin Lysis Time

1. To 0.5 ml. of 0.1 M. sodium oxalate add 4.5 ml. whole blood; use conical centrifuge tube which has been previously chilled in ice.

2. Take specimen to laboratory in melting ice bath.

3. Centrifuge at 3,000 rpm (in International Centrifuge) for 10 minutes in a centrifuge bucket which has been chilled in refrigerator prior to use.

4. Separate plasma into iced test tube.

Note: Steps 5-11 should be carried out in duplicate.

5. Add 0.5 ml. of plasma to 9 ml. distilled water; bring pH of solution to pH 5.2 - 5.4 by adding 0.1% acetic acid. Approximately 0.1 ml. acetic acid is usually required. Check in pH meter.

6. Shake solution thoroughly, then refrigerate tube at 4^{o}C. for 30 minutes.

7. Remove from refrigerator; white precipitate should be visible; this is the euglobulin precipitate.

8. Carefully decant the supernatant and break up the particles with a glass stirring rod.

9. Add 0.5 ml. borate solution (9 gm. NaCl + 1 gm. NaBorate + distilled water qs to 1 liter, pH = 9.0) to precipitate and be sure it dissolves fully before next step.

10. Place tube in water bath at 37^{o}C. and add 0.5 ml. of .025M calcium chloride solution, starting stopwatch simultaneously. Shake gently and replace in water bath. Record clotting time of solution.

11. Remove tube from water bath at 10 minute intervals and observe for lysis. Endpoint is total lysis of clot; i.e., when whole clot becomes fluid. Do not carry observations beyond 300 minutes.

Interpretation:

The euglobulin precipitate contains the bulk of the plasma fibrinolytic activity but little plasma inhibitor activity. Normal individuals should show complete lysis of the clot in 200-300 minutes. Times beyond 300 minutes indicate diminished activity. Times between 100 and 300 minutes indicate enhanced activity.

Times below 100 minutes indicate rather marked enhancement of fibrinolytic activity. Error is introduced, however, if the plasma fibrinogen level is diminished sharply. If fibrinogen is low, lysis time will be shortened for this reason. In patients with times below 100 minutes in whom question of this possibility exists, a qualitative or quantitative fibronogen estimation should be carried out on the same blood sample by the blood chemistry section of the laboratory department.

Whole Blood Clot Lysis

1. Draw approximately 5 ml. of whole blood.

2. Immediately place 2 ml. into each of two 75 x 100 mm. test tubes. Stopper tightly.

3. Incubate in 37°C. water bath.

4. Observe at 1 hour, and at 24 hours for indication of clot lysis.

Interpretation:

Unless there is bacterial contamination, clots undergo minimal lysis in a 24 hr. period. Dissolution of the clot during the 24 hour period indicates excessive fibronolytic activity.

Semiquantitation can be achieved with this test by (1) determining the hematocrit of the original blood sample, (2) determining the "hematocrit" of the serum following clot lysis.

$$\frac{\text{Post-lysis serum "hematocrit"}}{\text{Pre-lysis (true) hematocrit}} \times 0.5 \times 100 = \text{less than } 15\% \text{ (normally)}$$

The preceding is dependent on release of red cells from the clot as a result of fibrinolysis. Therefore do not unduly disturb any remaining clot when aspirating the sample for testing. Normally, a clot releases less than 15% during a 24 hour period.

FACTORS V AND VII

Factor V and Factor VII deficiency are primarily congenital defects, and their clinical pictures may bear a striking resemblance to true hemophilia.

In the second phase of coagulation, thromboplastin in the presence of Factor V, Factor VII, and calcium converts prothrombin to thrombin. The calcium is necessary for the action of Factor VII. Deficiencies of either prothrombin, Factor V or Factor VII, will result in a prolongation of the clotting time and the prothrombin time. This immediately places the difficulty in phase 2 rather than phase 1 (Figure 1). This is true because, in doing the prothrombin time, one does not depend upon thromboplastin generation, but supplies thromboplastin in doing the test. For this reason the prothrombin time is normal when the defect is with thromboplastin generation. When both the clotting time and the prothrombin time are prolonged, the most common deficiency is of Factor VII.

The properties of prothrombin, Factor V and Factor VII lend themselves to identification by both simple and more difficult laboratory methods. Factor V is not present in normal 48 hour serum. Factor VII and prothrombin are absorbed when normal plasma is treated with $BaSO_4$ (Table 9). The clotting defects may be corrected if $BaSO_4$ treated plasma and 48 hour serum are added to the unknown samples of blood to be tested. The results will be as follows:

	Normal $BaSO_4$ treated plasma	Normal 48 hour serum
Factor V deficiency	corrected	not corrected
Factor VII deficiency	not corrected	corrected
Prothrombin deficiency	not corrected	not corrected

A second procedure that can be used to differentiate is the two-stage prothrombin time. This consists in diluting plasma, after it has been defibrinated with a minute amount of thrombin and converting all the prothrombin to thrombin by adding thromboplastin and calcium. The thrombin is determined by the speed with which it coagulates a fixed amount of fibrinogen. Prothrombin is measured in units, one unit being the amount required to form one unit of thrombin; the latter is the quantity which will cause clotting of 1 cc. of fibrinogen solution in 15 seconds. The normal amount is 300 units/cc. or 100%. The original method did not eliminate either of the plasma accelerator factors as variables. A subsequent modification of the test eliminates Factor V as a variable by adding Factor V in the form of diluted beef serum (Ware-Seegars modification).

If the original and the modified technique of the two stage prothrombin determination are performed on the same sample of blood, deficiency of Factor V can be determined.

	Original	Modified
Prothrombin deficiency	delayed	delayed
Factor V deficiency	delayed	normal
Factor VII deficiency	delayed	delayed

Another point of differentiation is that in Factor VII deficiency, the prothrombin consumption is normal. This cannot be fully explained on the basis of the present knowledge concerning this factor. It has been postulated that the reason for the normal prothrombin consumption time is that the role of Factor VII lies in the initial phases of clotting, and its effects do not extend to the time when it will affect prothrombin consumption.

Calcium

Deficiency of calcium is never of clinical significance insofar as the clotting mechanism is concerned. A patient would die of other manifestations of hypocalcemia before the clotting mechanism became defective.

Fibrinogen

The loss of a peptide (or peptides) is now generally accepted as occurring during transformation of fibrinogen to fibrin.

Afibrinogenemia and hypofibrinogenemia are comparatively rare conditions; both may be congenital. Hypofibrinogenemia may accompany severe infections, burns, poor gastrointestinal absorption of proteins, certain malignancies, pernicious anemia, scurvy, pellagra, severe liver damage, and premature separation of the placenta.

Complete absence of fibrinogen results in complete incoagulability of the blood. Bleeding times may be normal or markedly prolonged. The coagulability of the blood cannot be induced by the addition of any agent other than fibrinogen.

Fibrinolysin

Fibrinogen is destroyed by an enzyme, fibrinolysin. At times hemorrhage following thoracic surgery or abruptio placentae is believed to be due to the release of excessive amounts of fibrinolysin into the circulation. Recently drugs have been developed which are fibrinolytic, i.e., dissolve blood clots. Those drugs may also induce fibrinogenopenia.

There is no completely satisfactory test for excessive fibrinolysin activity in the plasma. It is not yet possible to differentiate between fibrinolysin itself, and an "activator" which converts profibrinolysin to fibrinolysin. As a practical laboratory exercise whole blood clot lysis is related to fibrinolysin activity.

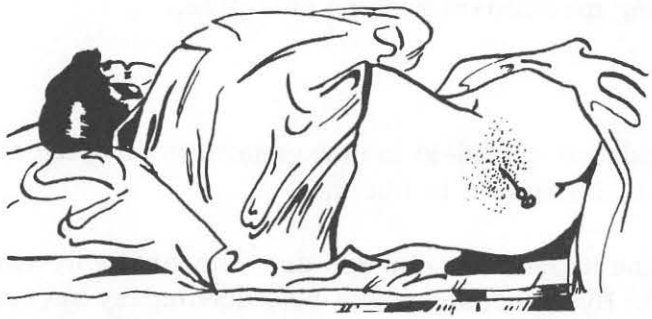
BONE MARROW ASPIRATION

Discussion

It is often necessary to obtain bone marrow specimens to properly interpret changes seen in peripheral blood, as the latter only mirrors the changes taking place in the bone marrow. Bone marrow examination also helps to distinguish different hematopoietic diseases such as aplastic anemia and aleukemic leukemia. It is very important to obtain bone marrow units on the smears before making a diagnosis. One is unable to make a diagnosis of aplasia, hypoplasia, or hyperplasia on a smear alone, but this can be done on a bone marrow section from a Vim-Silverman needle (or other type of) biopsy.

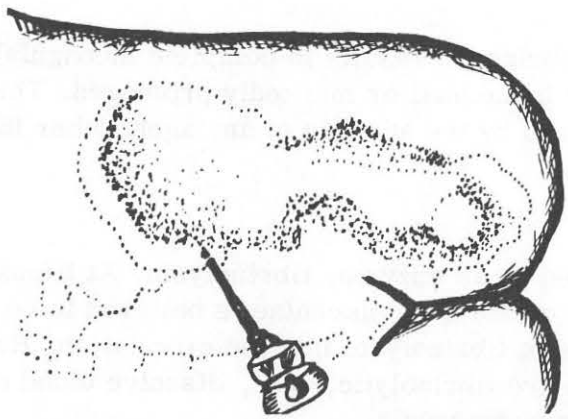
The posterior iliac crest procedure is utilized in the laboratories of the U.S. Naval Medical School. This technique is highly recommended. The sketch below illustrates the essential feature of the technique.

A.



A. Position of patient and usual direction of insertion of biopsy needle.

B.



B. Outline of ilium, showing usual site for introducing needle through posterior iliac crest. Tip of needle is just inside cortex, and obturator has been removed. Needle is directed towards anterior superior iliac spine as it is inserted.

Bone Marrow Aspiration Procedure

1. Have patient lie (on bed, table, or stretcher) on his side, with his knees drawn up, and back flexed. The back is positioned at the edge of the table.

2. The lumbosacral area is palpated to locate the posterior iliac crest, and the medial and lateral borders of the latter may be outlined on the skin with a cotton-tipped applicator and ink or dye. This is helpful in obese patients.

3. The posterior superior iliac spine is located and a point approximately 1 cm. cephalad to it on the iliac crest is marked with an "X" by fingernail impression.

4. The skin is surgically cleansed in the usual manner using Phisohex (or other suitable soap) followed by 70% alcohol (or other suitable antiseptic). Drape sterile towels over the back leaving only the surgically cleansed skin exposed to avoid contamination.

5. A skin wheal is raised by intradermal injection of a local anesthetic such as Procaine at the "X" mark using a small 26-gauge needle. Using a longer 23-gauge needle the subcutaneous tissue and periosteum are infiltrated with the anesthetic. It is well to infiltrate the periosteum over a circular area approximately 2 cm. in diameter.

6. When satisfactory anesthesia has been obtained, a marrow aspiration needle such as the Robertson (with stylet in place) is pressed through the cortical bone by using a steady pressure combined with rotary twisting of the needle. A noticeable "give" in resistance to the needle indicates the cortex has been penetrated, and the needle need be advanced only a few millimeters further.

7. Prepare a 20 or 30 ml. syringe by rinsing it with approximately 1 cc. of heparin (1 mg % in saline) and expel all but approximately 0.5 cc. Heparin sodium has been satisfactory as the anticoagulant. For example, from a stock bottle of 10,000 USP units per cc (100 mgm per cc) one cc is diluted to 100 ccs with saline for marrow use (one mgm per cc) and is kept refrigerated.

8. Remove the stylet from the marrow-needle, attach the syringe, and give a quick, strong pull on the plunger. (Note: to avoid startling the patient it is well to inform him that he may experience discomfort or transient pain at this stage, the pain usually being referred to the leg.) Additional aspiration is contraindicated since it only dilutes the marrow specimen with unwanted peripheral blood due to rupture of small marrow blood vessels. Excess aspirate also leads to unwanted clotting in the specimen.

9. If additional material is wanted, slightly advance or retract the needle while rotating it 180 degrees. Now additional aspiration will provide additional marrow.

10. Rotate the petri dish into which the excess heparin had been placed in order to wet the surface with heparin; discard any excess heparin.

11. Expel the syringe contents (marrow particles)into the petri dish. Bacteriologic cultures may also be made at this time.

12. Back-light the petri dish with a microscope lamp, holding the dish at a 20-30 degree angle to the table top and lamp. Rotate the dish. Minute pale white glistening flecks will be seen as the blood film flows across the glass surface of the petri dish. These are the bone marrow "units" wanted for smearing.

13. With a slender tipped pipet, aspirate a unit and deposit it on a clean coverslip, removing excess blood or plasm by aspiration. Place another clean coverslip over it, press gently to spread the unit and then quickly draw the coverslips apart. Fan the coverslips dry, stain and then counterstain with a 1:10 solution of Giemsa stain for ten minutes.

14. If marrow units are not obtained by aspiration on examination of the petri dish contents, proceed with a Vim-Silverman needle biopsy of the marrow. With obturator in place, the V-S needle is twisted through the cortex, being aimed towards the anterior superior iliac spine. Stop when the needle and obturator pass through the cortex. Remove the obturator and insert the biopsy blades the length of the needle. With firm pressure, advance them the remainder of their length. As the cutting blades advance, they are forced apart by the increasingly thicker wedge of marrow caught between them. Hold the blades firmly while advancing the needle cannula more deeply over the blades until it stops. It is usually impossible to advance the cannula completely due to the thickness of the wedge of bone held in the biopsy blades. Give both blades and cannula a twist to separate the biopsy specimen from the bony trabecula of the marrow. Hold the pieces firmly together while withdrawing the blades and the cannula as one unit with its contained marrow biopsy plug.

Push the bony plug out of the blades with the needle previously used for anesthesia. The thick bone plug cannot be satisfactorily drawn through the cannula by trying to remove the cutting blades through the cannula.

Drop the bone plug into freshly prepared acidified Zenker's solution. If allowed to stand 4 to 12 hours decalcification will also take place. Wash the plug for an equal time in running water, process the plug for histologic examination, and stain with H & E or Wright's stain (or other). Impression smears on coverslips may be made by touching the plug to them before placing it in Zenker's fixative.

15. When the aspiration or biopsy has been completed and the cannula or needle has been withdrawn, place a sterile gauze 4 x 4 over the puncture site. Instruct the patient to maintain pressure on the site by holding the gauze, or lying on it for at least 15 minutes. Maintenance of pressure is important to minimize the chance of hematoma formation.

16. At the time of bone marrow aspiration prepare fresh peripheral blood smears on coverslips and stain with Wright's. Mount a peripheral smear coverslip adjacent to the bone marrow coverslip on a 1 x 3 micro slide. Bone marrow and peripheral blood are thus conveniently adjacent for the pathologist's examination and comparison.

BONE MARROW DIFFERENTIAL COUNT

The erythrogranulocytic ratio in the bone marrow varies from 1:2 to 1:6 usually being 1:3-4. First examine the smear under low power to check cellularity and note the presence of megakaryocytes. It is good training for technicians to do marrow counts. However, Custer doubts the value of counts except under well controlled conditions, stating that when deviations from the normal do occur, it is seldom necessary to do a count to make it apparent.

The following chart is presented as a guide to a so-called normal bone marrow count.

BONE MARROW DIFFERENTIAL COUNT

Cell Type	Range in Per Cent
Undifferential cells	0.0
Blasts	0 -3.5
Progranulocyte	0.5 - 5.0
Myelocytes	
Neutrophil	7.0 - 34.6
Eosinophil	0.3 - 3.0
Basophil	0 - 0.5
Metamyelocytes	
Neutrophil	14.8 - 33.0
Eosinophil	0.3 - 3.7
Basophil	0 - 0.3
Band	
Neutrophil	15 - 30
Eosinophil	0.2 - 2.0
Basophil	0 - 0.3
Segmented cells	
Neutrophil	3.0 - 19.8
Eosinophil	0.1 - 3.0
Basophil	0 - 1.0
Lymphocytes	5 - 20
Monocytes	0.5 - 4
Plasmacytes	0 - 1.2
Megakaryoblasts	0 - 0.25
Rubriblasts	0 - 0.5
Prorubricytes	0 - 0.5
Rubricytes	5 - 25
Metarubricytes	10 - 20

WRIGHT-GIEMSA STAIN TECHNIQUE FOR BONE MARROW PREPARATIONS

Reagents

Wright's Stain-preparation previously described.

Giemsa-stain available as a stock solution; dilute 1 part of Giemsa stock with 9 parts of pH 7.0 aqueous buffer solution (buffer salts and instructions for use commonly are supplied with the stock solution). Other forms of Giemsa stock are available, in which case follow the appropriate technique.

Procedure:

1. Overlay 10 drops of Wright's stain on marrow smear. Let stand 1 minute.
2. Add 10 drops of pH 6.8 aqueous buffer; let stand 3 minutes. Blow gently on surface to mix until metallic sheen forms.
3. Rinse slide with distilled water; drain excess from slide.
4. Flood smear with Giemsa stain (10% dilution); let stand 10 minutes or longer. Evaluate time by degree of staining of the granules in the granulocytic series.
5. Rinse with distilled water, blot and air dry.
6. Mount slide or coverslip with permount. Examine.

N.B. The preparation and use of stains is still a highly individualized experience. Modify the technique according to your own experience to produce the desired result by varying the staining times, and/or buffer pH.

THE BLOOD PICTURE IN INFANTS AND CHILDREN

The hematopoietic system in infants and children is very sensitive, tending to react vigorously to slight stimuli. A mild infection may produce a marked shift to the left, and increased numbers of reticulocytes and nucleated red cells appear in the peripheral blood upon slight provocation. There may be a marked relative or absolute lymphocytosis in conditions that would scarcely change the blood picture of an adult.

Normally at birth the leukocytic picture is neutrophilic with some tendency towards immaturity. After one week, the neutrophil-lymphocyte ratio is inverted, and the normal adult ratio is reached sometime between the sixth and twelfth year.

Thrombocytes are variable in numbers, but rarely are they decreased.

Reticulocytes from birth to four months range from 1 per cent to 5 per cent, and in order children 0.5 per cent to 4 per cent. Polychromasia and anisocytosis are normal up to about six months.

Normal hemoglobin levels range about 13 grams until adolescence when adult levels are reached.

SOME CONDITIONS HAVING IMPORTANT BLOOD FINDINGS

Acute Hemolytic Anemia

This condition is characterized by rapid destruction of blood accompanied by chills, fever, weakness, nausea, vomiting, and pain in the back, abdomen and extremities. Pallor and jaundice develop rapidly, and the urine and feces become orange-colored due to urobilinogen. There is no bile in the urine, but hemoglobinuria may occur. The liver and spleen enlarge. Anuria may develop. There are many causes, some of which follow: malaria, blackwater fever, bartonelliasis, Welch bacillus, hemolytic streptococcus, phenylhydrazine, thiouracil, plasmoquin sulfonamides, arseniurreted hydrogen, trinitrotoluene, dinitrobenzol, benzene, favism, snake venom, extensive burns, incompatible blood transfusion, and autohemolysins.

Another group of the acute hemolytic anemias are the paroxysmal hemoglobinurias. These are the paroxysmal cold hemoglobinuria, paroxysmal nocturnal hemoglobinuria (Marchiafava-Micheli syndrome), march hemoglobinuria, and paralytic myohemoglobinuria. Paroxysmal cold hemoglobinuria is a condition characterized by the sudden passage of hemoglobin in the urine following local or general exposure to cold. The phenomenon is due to a peculiar cold agglutinin. Syphilis, congenital or acquired, appears to be the fundamental cause though the Wassermann reaction is not always positive. The Donath-Landsteiner test or modifications confirm the presence of the cold hemolysin. Paroxysmal nocturnal hemoglobinuria is an uncommon disorder of insidious onset and chronic course characterized by signs of hemolytic anemia and marked by attacks of hemoglobinuria which occur chiefly when asleep, whether during the day or at night. Carbon dioxide becomes more concentrated during sleep with a resulting lowering of the hydrogen - ion concentration of the blood. The hemolysis depends on the presence of cells sensitive to hemolysis by a thermolabile component of normal serum which is affected by changes in the hydrogen ion concentration. The confirmatory test is the acidified serum test of Ham. March hemoglobinuria is rare and does not cause anemia. It is observed after prolonged walking or running. The cause is unknown. In paralytic myohemoglobinuria, the disorder is attributed to the sudden release of lactic acid from the excessive amounts of glycogen which have accumulated in

the muscles. The lactic acid damages the muscles and allows the myohemoglobin to escape into the blood and urine. It is rare and no anemia is associated with it.

The blood picture in the acute hemolytic anemias is usually that of a profound normocytic, normochromic anemia with a high reticulocyte count. There is usually a leukocytosis, which may be marked, with a left shift of the neutrophils. The fragility test is usually normal, although some cases with recurring attacks show increased fragility of the red cells and are difficult to differentiate from congenital hemolytic jaundice during a crisis. Differentiation is by the direct Coomb's test which is positive in acquired but not in congenital hemolytic anemia. The thrombocyte count is usually increased. The bleeding time and coagulation time are normal.

Agranulocytosis

This is a disease characterized by the absence or marked decrease in the number of neutrophils in the differential count due to a sensitivity to certain drugs, among them aminopyrine, organic arsenicals, sulfonamides, dinitrophenol, thiouracil, benzene, and gold salts. Those drugs having a benzene ring with an attached amine are considered the most dangerous. The bone marrow may show a decrease to absence of metamyelocytes, band and segmented neutrophils, with an increase in myeloblasts, which is interpreted as a maturation arrest; or if late in the disease the bone marrow may be normal. There is usually a low white count, no anemia, normal thrombocyte count, normal bleeding and coagulation time, and increased sedimentation time. The patient may suffer from fever, weakness, and gangrenous ulceration of the throat.

Aplastic Anemia

This is a severe, usually normocytic, normochromic anemia characterized by marked diminution, or complete absence of reticulocytes, nucleated erythrocytes, and polychromatophilic red cells of the blood and bone marrow. The granular leukocytes and thrombocytes are markedly decreased with delayed clot retraction and prolonged bleeding time. All series of the bone marrow, erythrocytic, granulocytic and thrombocytic, are either markedly decreased in number, or if present, the ability to mobilize is impaired. The cause may be idiopathic or due to poisoning by drugs containing the benzene ring, overdosage of x-ray, or radium, among other causes.

Congenital Hemolytic Anemia

This is a chronic type of hemolytic anemia due to an inherited anomaly of the red cells which tend to microspherocytosis and increased fragility in hypotonic

salt solution. There are recurrent attacks of hemolytic jaundice with splenomegaly. Rouleaux formation is bizarre in wet preparations of blood. The fragility test shows beginning hemolysis between 0.5 and 0.8 per cent sodium chloride and may be complete at 0.36 per cent. Reticulocytes are greatly increased.

Erythremia or Idiopathic Polycythemia Vera

Polycythemia vera is characterized by a definite increase in red cells as well as blood volume. Hemoglobin may vary from 17-24 gms./100 ml., erythrocytes 6-10.5 million/cu. mm., hematocrit 50-80 per cent, and total blood volume 75-140 ml./Kg. Bone marrow examination shows increase in the granulocytic as well as the erythrocytic series, the latter being mostly rubricytes and metarubricytes. Many authors regard this disease as being a counterpart of leukemia, in that it represents a malignant neoplasia of red blood cells. A very definite percentage of these cases terminate as a granulocytic leukemia.

Erythroblastosis Fetalis, or Hemolytic Anemia of the Newborn

This is a hemolytic anemia which occurs late in fetal life or shortly after birth and is characterized by a severe macrocytic anemia with pallor and jaundice and sometimes edema. The peripheral blood is flooded with nucleated red cells and reticulocytes which come from hyperplastic centers of erythropoiesis in the bone marrow, liver, and spleen in an attempt to compensate for the excessive red cell destruction. The white count is also increased with a shift to the left. Thrombocytes may be reduced. The direct Coomb's test is positive. The hemolysis in the majority of cases is due to the immunizing of the mother, who is Rh negative, to the red cells of the fetus, who is Rh positive. The agglutinin of the mother's serum then produces a hemolytic action on the fetal red cells. The mother's milk may also contain this agglutinin. About 85 per cent of the population are Rh positive and 15 per cent Rh negative. Since only about one of fifty Rh negative individuals are readily sensitized and then so slowly that the first offspring escapes, and since the fetal Rh positive red cells must break through the placental barrier to sensitize the mother, it is estimated that only about 1 in 200 or 300 pregnancies result in erythroblastotic babies. Treatment is by transfusions of properly matched Rh negative whole blood. Erythroblastosis may also be due to blood group factors other than Rh; for a discussion of these blood groups see U. S. Naval Medical School manual of Blood Bank Procedures.

Infectious Mononucleosis

This is a rather common disease, usually characterized by fever, sore throat, enlarged lymph nodes and spleen. The leukocyte count varies from 6,000 to 40,000 with 50 to 90 per cent lymphocytes. The lymphocytes are atypical. They vary in characteristics from those of prolymphocytes to those of medium size lymphocytes with bean-shaped nuclei and dark to light blue cytoplasm.

Vacuoles are often present in the cytoplasm and sometimes in the nucleus. The red cell count and thrombocyte count are usually normal. The serum agglutinates sheep cells in high dilution in 80-90 per cent of the cases. (Paul-Bunnell test) (Davidsohn Test). A disease similar to this is seen in children and called infectious lymphocytosis because of a very high white count reaching 50,000 to 90,000 with absolute increase in lymphocyte count. No atypical lymphocytes are seen and the Davidsohn test is negative. Refer to Serology manual for details of differential serologic tests for infectious mononucleosis.

Leukemias

These are fatal diseases characterized by uncontrolled proliferation of leukopoietic tissue. The cells may be granulocytic, lymphocytic, or monocytic. The disease may be acute, subacute, or chronic. The leukocyte count is usually high, and immature types are present. The acute type, in particular is characterized by the presence in the blood of many "blast" cells. Anemia is present and in the acute phase usually is severe. Thrombocytopenia with tendency to hemorrhage usually occurs. Not all leukemias have a high circulating white count even though the leukopoietic tissue of the bone marrow is hyperplastic. The white count may be low or normal, and the leukemia is then called aleukemic, leukopenic or subleukemic. Atypical blood pictures are common, and an examination of the bone marrow is necessary to establish a diagnosis.

Leukemoid Reactions

These are blood conditions that closely resemble, or are indistinguishable from, those of the various types of leukemia. Leukemic changes in the tissues of the body are absent. These reactions have been found occasionally in various infections, chemical and drug poisonings, severe burns, severe hemorrhages or sudden hemolysis of blood and in malignant metastases to bone. The clinical history, subsequent course, alkaline phosphatase reaction of the granulocytic cells, and bone marrow examination are necessary for differential diagnosis.

Thalassemia (Mediterranean Anemia)

This is an inherited type of anemia seen in Italians, Greeks, Syrians, and Armenians. The red cells are thinner than normal and take the form of target cells or are otherwise distorted due to folding of the thin edges. Those individuals with a homozygous inheritance usually die in childhood from a severe anemia called thalassemia major (Cooley's anemia), while those with a heterozygous inheritance of the red cell anomally, thalassemia minor, usually go through life with a hypchromic microcytic anemia or compensate for the low content of hemoglobin in the thin cells by developing a red count that is higher than normal. Thalassemia major is characterized by the presence of hemoglobin "F" in large amounts. Hgb A-2 is present in thalassemia minor, as seen on starch block by electrophoresis.

Microcytic Hypochromic Anemia

This is a common type of anemia in which the red cells are smaller than normal and poorly filled with hemoglobin causing an exaggeration of the central pallor in a stained smear. The cause is an iron deficiency, due either to chronic blood loss, poor diet, poor absorption or poor utilization of iron.

Myelophthisic Anemia

This is a type of anemia associated with space-occupying lesions of the bone marrow, among which are metastatic carcinoma, multiple myeloma, myelosclerosis, marble bone disease, Hodgkin's disease, and lipoid hystiocytosis. The anemia is variable in degree and may be normocytic or macrocytic. There are many nucleated red cells in the smear and myelocytes, progranulocytes, and occasionally myeloblasts are present. The leukocyte count may be low, normal, or high. As in other instances, bone marrow examination is required to establish the diagnosis.

Pernicious Anemia

This is a deficiency disease with a macrocytic type of anemia due to lack of an intrinsic factor formed in the stomach mucosa which is necessary for the absorption of vitamin B-12. Normally this erythrocyte maturation factor is formed in excess and stored in the liver, so that normal liver, injected into pernicious anemia patients supplies the deficiency. The blood is characterized by an increase in size of the red cells, which are usually well filled with hemoglobin. A characteristic type of nucleated erythrocyte may be present. This is the rubriblast, of the "pernicious anemia type." The thrombocyte and leukocyte counts are usually reduced. There may be giant thrombocytes. The neutrophils are characteristically of the hypersegmented type. During relapse, bone marrow smears show many rubriblasts of the pernicious anemia type (megaloblasts). During vitamin B-12 or liver therapy, a reticulocytosis is produced. Characteristic findings in the patient are the following: lack of free hydrochloric acid in the stomach contents, glossitis, and spinal cord changes. Macrocytic anemia is also sometimes seen in sprue, pregnancy, liver disease, carcinoma of the stomach, fish tapeworm infestation, intestinal resection or strictures, and achrestic anemia. The latter disease is a progressive macrocytic anemia, usually without spinal cord symptoms or glossitis, and with free hydrochloric acid in the gastic juice. It does not respond to liver due to inability of the bone marrow to utilize the erythrocyte maturation factor. The other diseases listed are also due to deficiencies, chiefly a lack of folic acid.

The radioactive Cobalt-60 Vitamin B-12 absorption test (Schilling test) is very helpful in establishing a diagnosis of pernicious anemia. Refer to the NMS Radioisotopes manual for details.

Primary Splenic Neutropenia and Primary Splenic Panhematopenia

These names have been applied to diseases in which the normal physiologic phagocytosis of circulating blood cells by the reticuloendothelium of the spleen becomes pathologically intensified. This produces some, or all, of the symptoms and findings of agranulocytosis, hemolytic anemia, and thrombocytopenia. Bone marrow elements are normal, except for hyperplasia. The spleen is enlarged, and splenectomy is effective therapy.

Sickle Cell Anemia

This is a hereditary anomaly of the erythrocytes, limited essentially to persons of Negro ancestry. The red cells take on a sickle shape when the oxygen tension is reduced. The sickle cell trait is found in about 7.3 per cent of negroes, but only about one out of forty with the trait develops anemia. The anemia is hemolytic, and there are nucleated red cells, polychromasia, stippling, and increased number of reticulocytes in the peripheral blood. There is leukocytosis and an increase in thrombocytes. Red cells may show increased resistance to hypotonic salt solution. Rouleaux formation is prevented due to the shape of the red cells, so that the sedimentation rate is low.

Thrombocytopenic Purpura

This disease is characterized by interstitial hemorrhages in the skin and mucous membranes. There may be gross bleeding from the mucous membranes, uterus, gastrointestinal tract, etc. The thrombocyte count is low due either to deficient production by the bone marrow, excessive destruction of thrombocytes by the spleen or excessive utilization of thrombocytes in preventing leakage from damaged capillaries. The bleeding time is prolonged, clot retraction is poor, capillary fragility increased, coagulation time normal, and thrombocyte count is usually under 60,000 if purpura is present. Anemia is proportional to the hemorrhage. Neutrophilia may also be present due to hemorrhage. Bone marrow aspiration usually shows a normal number and morphologic appearance of the megakarocytes in the idiopathic type. In the secondary type the megakarocytes may show changes from the effects of the toxins, drugs, chemicals, etc. The toxic changes include lack of normal granules, vacuolization or hyalinization of the cytoplasm and small, round pyknotic nuclei.

ABNORMAL HEMOGLOBINS

The normal red cell has the shape of a biconcave disc. Characteristic shape anomalies such as spherocytosis, leptocytosis (target cells), drepanocytosis (sickle cells), elliptocytosis and acanthrocytosis (burr-like cells) have been noted in hereditary diseases, and are associated with hemoglobin anomalies.

Hemoglobin is composed of an iron-porphyrin complex (heme) in combination with a protein moiety, globin. Heme imparts red color to the molecule. The globin fraction exhibits species specificity. The dissimilarities of human hemoglobin types reside in the protein part and are due to amino acid differences. They have been uncovered by the application of methods generally used for the characterization of proteins, such as electrophoresis, resistance to denaturing agents, solubility studies, spectroscopic and immunologic behavior.

Terminology of Hemoglobins:

Normal adult	**A (A_2 seen on starch block)**
Fetal	**F**
1st Pathologic (sickle cell)	**S**
2nd " hemoglobin	**C**
3rd " "	**D**
4th " "	**E**
5th " "	**G**
6th " "	**H**
7th " "	**I**
8th " "	**J**

Mobility	H	I	J	A	F	G	S	D	E	C
Paper electrophoresis pH8.6	fastest	same as H	faster than A, slower than H	faster than F, slower than J	faster than S, slower than A	between A&S	faster than C, slower than A	like S	between S&C	slowest

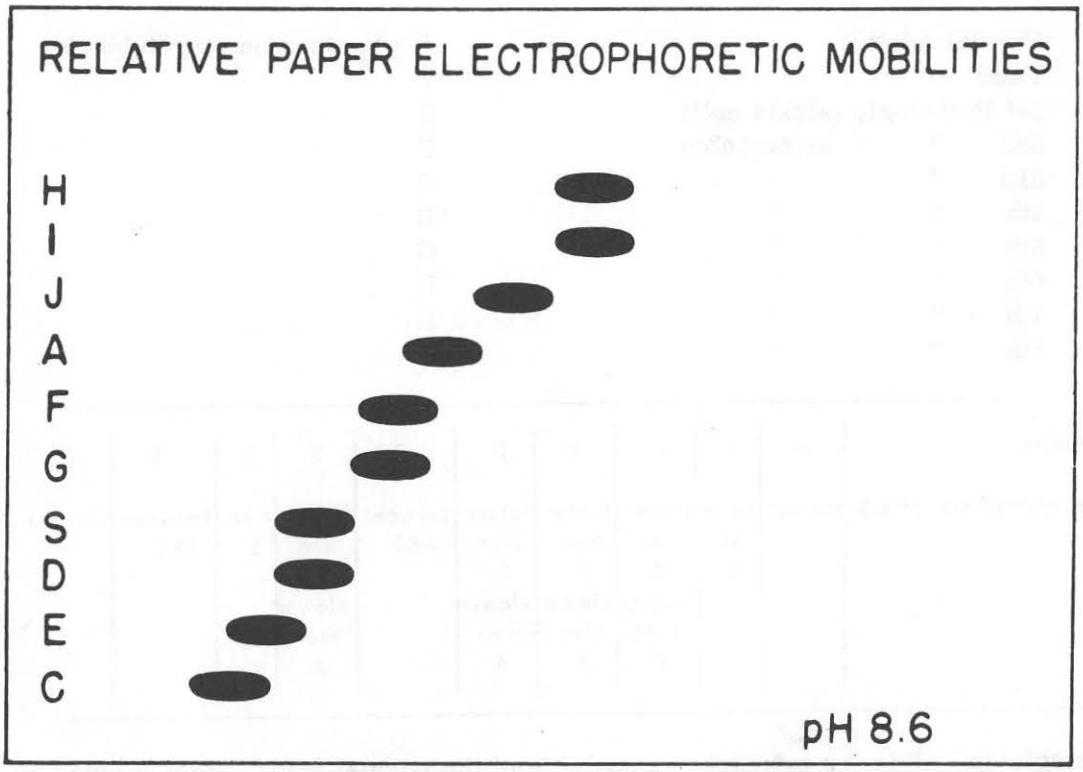
Hemoglobin "A" is the principle hemoglobin of the normal adult. Hemoglobin "F" is the hemoglobin formed by the fetus in utero and present for some time after birth; it is ordinarily not detectable at age 1 year. Hemoglobin "F" characterizes Thalassemia major, hemoglobin "S", or sickle cell hemoglobin, was the first abnormal hemoglobin to be discovered; since then, over 30 distinct hemoglobin species have been described. The most important of the abnormal hemoglobins are "S" and "C" in the Negro races, and hemoglobin "E" in the southern Asian peoples (Thailand).

At pH 8.6 hemoglobin "C" moves slowest, hemoglobin "H" and "I" fastest, and hemoglobin "S" has an intermediate mobility. Fetal hemoglobin cannot be distinguished from normal adult hemoglobin by electrophoresis, nor can hemoglobin "S" from hemoglobin "D". Hemoglobin "F" can be separated from hemoglobin "A" by the alkali denaturation technic.

Electrophoresis on paper strips is capable of delineating proteins present in a concentration of 20% or more.

Electrophoresis on starch blocks is sensitive to concentrations of hemoglobin in the range of 1% of the total. Hemoglobin "A_2" is demonstrated by this method; in other respects it produces results similar to paper electrophoresis.

Electrophoresis in thin sheets of agar buffered at pH 6.2 clearly separates hemoglobin "F", and also differentiates hemoglobin "D" from "S".



In paper chromatography, one drop of hemolysate (10 gm. % hemoglobin solution) is placed in the middle of a strip of Whattman No. 3 MM filter paper, previously moistened with veronal buffer pH 8.6, ionic strength 0.06. Staining of proteins with bromophenol blue is recommended. Since pH of buffer is on the alkaline side of the isoelectric points of these proteins, hemoglobin "H" and "I" moves fasttest, hemoglobin "C" slowest, hemoglobin "S" intermediate.

Alkali Denaturation Technic

When oxyhemoglobin is exposed to alkali, a brownish material, alkaline globin hematin, is formed. The reaction involves oxidation of the heme iron to the ferric state as well as denaturation of the globin. The most important factors influencing this reaction are pH, temperature, and time of exposure. At pH 12.7 all the "non-resistant" hemoglobins are completely denatured within one minute. If exposure time is extended, the "resistant" hemoglobin also will become partly denatured. Since all the non-resistant pigments are denatured within one minute, the "one minute denaturation value" represents the percentage of alkali-resistant pigment minus the small quantity of this fraction (5%) which has been destroyed during the test. The total percentage can be computed from the one minute denaturation value, corrected by addition of 5% of this value. The method consists of exposing 0.1 cc. of approximately 10 gm.% stroma free hemolysate to 1.6 cc. of N/12 NaOH (pH 12.7) for exactly one minute at 20°C. The denaturation process is then interrupted by addition of 3.4 cc. of reagent (1 cc. of 10 N HCl to 400 cc. half saturated ammonium sulfate) which precipitates the denatured chromogens and simultaneously stops the reaction by lowering the pH. Filter and read the amount of unaltered hemoglobin and express as a percentage of the initial amount of hemoglobin. Readings of 0.5 to 1.7% are obtained in normal adults due to split products of the denatured proteins which go into solution and possibly to minute amounts of "resistant" hemoglobin present in adult blood. Values over 2% definitely indicate presence of an abnormal mount of alkali-resistant pigment. Children to the age of 2 years, may have a value over 2% normally.

Of the known hemoglobin types, only fetal hemoglobin exhibits resistance to alkali. Whether the alkali-resistant pigment fraction, which appears in sickle cell anemia, thalassemia and other conditions, is in all aspects identical with the similarly behaving compound found in cord blood, or whether it is only "fetal-like" remains a moot question. At present, it seems most advantageous to designate any alkali-resistant human red cell pigment as hemoglobin "F."

The dissimilarities in the various human pigments are considered at present to be caused essentially by differences in amino acid and in the folding and coiling of the polypeptide chains which constitute the globin molecule. Since the hemoglobin abnormalities are genetically controlled, one must visualize the specific gene action as directing these particular aspects of globin synthesis. The assumption that the determinants for pathologic hemoglobin types are alleles of the genes for hemoglobin "A", provides the most satisfactory explanation of the basic genetic features of these diseases.

It is suggested that the various components demonstrated in hemolysates be reported in the sequence of their quantitative representation. Thus the formula S + A + F indicates that hemoglobin "S" is the major and hemoglobin "A" the larger of the two minor components.

Sickle Cell Disease (Disorders associated with hemoglobin S)

A positive sickle cell test merely proves the presence of S hemoglobin. Hemoglobin analysis permits a sharp classification of the syndromes.

1. Sickle cell trait (sicklemia) is characterized by A + S pattern and represents the heterozygous state for the hemoglobin S gene. The amount of S pigment is greater than 20%, but less than 50% of the total hemoglobin.

2. Sickle cell anemia represents the homozygous state for the hemoglobin S gene. Only rarely is hemoglobin S the sole component in the hemolysate. Usually a non-S fraction is demonstrable which varies from 2 to 24% ;and is entirely composed of alkali-resistant hemoglobin F. The S or the S + F pattern is characteristic.

3. Hemoglobin C - sickle cell disease represents the double heterozygous state for these two abnormal hemoglobins - C + S or C + S + F pattern.

4. Hemoglobin S - thalassemia (micro-drepanocytic) disease represents the double heterozygous state for the hemoglobin S and the thalassemia genes - S + A + F or S + A pattern. Both features of sickle cell and thalassemia are seen.

5. Hemoglobin D - sickle cell disease represents the double heterozygous state for two abnormal pigments D + S pattern which is indistinguishable from A + D pattern. The hemoglobin A + D trait reported in whites only (2 cases), gives a negative sickling test although electrophoretically it is indistinguishable from the sickle cell trait, except on agar.

Disorders associated with hemoglobin C

Target cells are regularly observed in all disorders associated with hemoglobin C but are not specific for the presence of this pigment. Marked targeting in a Negro patient is a definite indication for hemoglobin analysis.

1. Hemoglobin C trait represents the heterozygous state for this abnormal pigment - A + C pattern. The percentage of C hemoglobin varies from 28 to 40%. No abnormality of red cells is present except for a variable degree of leptocytosis which may be associated with a decreased osmotic fragility.

2. Hemoglobin C disease represents the homozygous state for hemoglobin C. Usually hemoglobin C is the sole component found in the hemolysate. Leptocytosis is very conspicuous, and the osmotic fragility is markedly decreased.

3. Hemoglobin C - thalassemia disease represents the double heterozygous state for the hemoglobin C and thalassemia genes. C + A and the C + A + F patterns are seen with hemoglobin C amounting to about 75%.

4. Hemoglobin C - sickle cell disease - see under sickle cell disease.

Hereditary disorders associated with hemoglobin F - Hemoglobin constitutes 50-90% of the red call pigment in the newborn. Within the first seven to twelve months of life, it is usually replaced by hemoglobin A or, in the hemoglobinopathies, by the respective abnormal pigments. Hemoglobin F has been detected in some of the thalassemic syndromes, in some with hereditary spherocytosis, and in those previously discussed.

1. Thalassemic syndromes. The common denominator is the production of structurally deficient, thin (leptocytic) erythrocytes. Hemoglobin analysis reveals 40 to almost 100% hemoglobin F in thalassemia major and none in thalassemia minor.

2. Hereditary spherocytosis. Occasionally patients may show hemoglobin F up to 8%.

3. It is now evident that thalassemic syndromes may be associated not only with hemoglobin F but also with hemoglobin S, C, or E.

Hereditary disorders with hemoglobin E

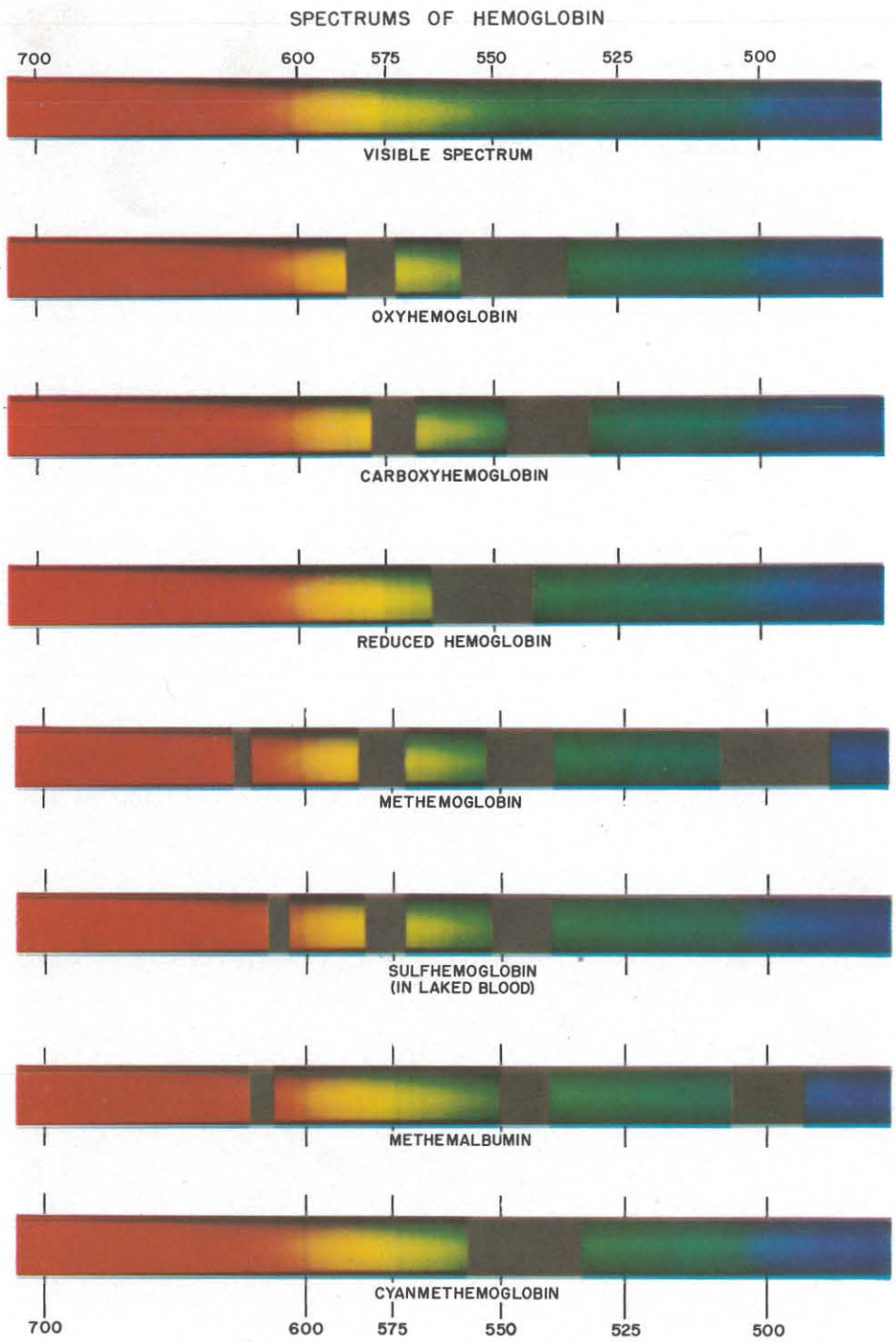
It is found in 13% of Thais and 6% of Indonesians.

Other abnormal hemoglobins

1. Hemoglobin G - rare with only two definite instances reported.

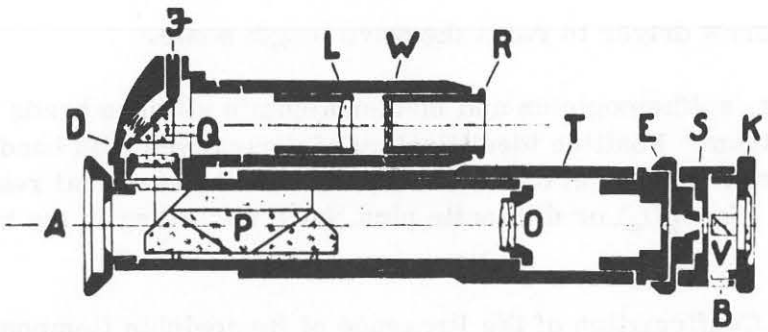
2. Hemoglobin H - rare with only report being that of three generations in one Negro family.

3. Hemoglobin J - rare with only report being that of two generations in one Negro family.


SPECTRUMS OF HEMOGLOBIN
700
600
575
550
525
500
VISIBLE SPECTRUM
OXYHEMOGLOBIN
CARBOXYHEMOGLOBIN
REDUCED HEMOGLOBIN
METHEMOGLOBIN
SULFHEMOGLOBIN
(IN LAKED BLOOD)
METHEMALBUMIN
CYANMETHEMOGLOBIN
700
600
575
550
525
500

IDENTIFICATION OF HEMOGLOBIN DERIVATIVES

Hand Spectroscope Adjustments



Identify:

A. Observer's eye
B. Window for comparison prism
D. Magnifier (wave length)
E. Knurled ring
Symmetrical adjustable slit jaws
F. Wave length scale adjusting screw
K. Knurled ring moves comparison prism
L. Wave length scale lens
O. Achromatic focusing magnifier O
P. Direct vision prism (Amici)
Q. Wave length scale prism
R. Adjustable sleeve (wave length)
S. Slit
T. Adjustable sleeve (slit)
V. Comparison prism
W. Wave length scale

Procedure

1. Adjust the slit for best definition by just barely opening it. You can permanently damage the spectroscope by jamming the jaws of the adjustable slit together too hard.

2. Focus the eye piece; this focuses the spectrum.

3. Focus the wave length tube.

4. Check the wave length calibration by any of the following methods:

   a. Neon light, incubator pilot light, 585 and 650 mu.
   b. Fluorescent light (mercury lines) 436 or 546 mu.
   c. Fraunhofer sodium line 589 mu. - (sun).

Use a small screw driver to reset the wave length scale.

Methemoglobin, sulfhemoglobin and methemalbumin all have bands in the red portion of the spectrum. Positive identification of derivatives with bands in the red is made by the selective removal of the absorption band by chemical reactions with cyanide, dithionite ($Na_2S_2O_4$) or dithionite plus NaOH according to the table below.

Reactions for Confirmation of the Presence of Hemoglobin Compounds of Clinical Significance with an Absorption Band in the Red Region of the Spectrum

Reagent	Methemoglobin	Hemoglobin Derivative Sulfhemoglobin	Methemalbumin
KCN	Disappear	Stable	Stable
$Na_2S_2O_4$	Disappear	Stable	Disappear
$Na_2S_2O_4$ + NaOH	Disappear	Disappear	Disappear

Neither HbCO or HbO_2 has bands in the red part of the spectrum but each has two almost identical bands in the orange and green portions. One band is at 578 and the other at 540 mu. In order to differentiate them, dithionite reagent is added to the unknown solution.

The bands of carboxyhemoglobin (Hb CO) persist in the presence of the reducing agent $Na_2S_2O_4$ (dithionite). Dithionite reacts with O_2 and lowers the oxygen tension of the solution, removing O_2 from HbO_2 to produce deoxygenated hemoglobin (reduced). This yields one band at 563 mu. instead of two (542 and 578 mu.).

Principal Reactions of Hemoglobin

1. $Hb + O_2 \rightleftharpoons HbO_2$. Oxyhemoglobin is a dissociable compound. Iron remains in ferrous state when O_2 is lost. Gain or loss of oxygen from hemoglobin depends upon the oxygen tension (partial pressure of oxygen and the pH of the surrounding solution).

2. $HbO_2 + Na_2S_2O_4 \longrightarrow Hb + NaHSO_3$ (plus other products). The dithionite

reacts with the oxygen in solution, reducing the O_2 tension to zero. As O_2 dissociates from HbO_2 it is removed by the reducing agent ($Na_2S_2O_4$).

3. $HbO_2 + Na_3Fe(CN)_6 \longrightarrow MHb + Na_4Fe(CN)_6$. (Oxidation, electron removal from iron in the hemoglobin by sodium ferricyanide).

$$\underset{(Hb)}{Fe^{++}} \longrightarrow \underset{(MHb)}{Fe^{+++}} + 1e \text{ (electron)}$$

4. $MHb + Na_2S_2O_4 \rightleftharpoons Hb + NaHSO_3 +$ other products (reduction, electron addition to iron in methemoglobin by the reducing agent dithionite).

$$\underset{(MHb)}{Fe^{+++}} + 1e \longrightarrow \underset{(Hb)}{Fe^{++}} \text{ (iron in methemoglobin)}$$

$$S^{-3} \longrightarrow S^{-5} \quad 2e \text{ (dithionite)}$$

5. $MHb + CN \longrightarrow MHbCN$ (cyanmethemoglobin)

6. $HbO_2 + CO \longrightarrow HbCO$. Carbon monoxide affinity for Hb is much greater than the affinity of O_2 for Hb; therefore, CO replaces O_2.

7. $HbCO + Na_2S_2O_4 \longrightarrow$ no reaction.

Note: The reducing compound $Na_2S_2O_4$ is called sodium dithionite, sodium hydrosulfite, lykopon, or sodium hyposulfite. It should not be confused with sodium thiosulfate which is $Na_2S_2O_3$.

SPINAL FLUID CELL COUNTS

Cerebro-spinal fluid is normally clear and colorless. It does not coagulate when permitted to stand at room temperature. In most instances, spinal fluid from healthy persons will contain no cells. Up to five lymphocytes per cc may be found and still be considered normal. When more than ten lymphocytes or any quantity of neutrophils is found, a pathologic condition is present. Erythrocytes are not found in spinal fluid unless there has been trauma at the time and site of the aspiration or unless there has been other bleeding into the cerebro-spinal fluid which bathes the brain and spinal cord.

Cerebro-spinal fluid sent to the laboratory for examination should be evaluated for its color, which is either completely absent (colorless), gray-green, red-yellow, or yellow-brown (xanthochromia). The appearance is reported as clear, faintly turbid, or grossly turbid.

CAUTION: Turbid specimens should always be treated with respect since they may have viable organisms in them. Even clear specimens may contain a virus, so cautious handling is always indicated. The presence of a coagulum (a thin fibrin clot) should always be noted and reported. Cell counts (erythrocyte and leukocyte) are usually desired.

Examination of cerebro-spinal fluid should always be made without delay; always within one hour after having been obtained. Cells present in the cerebro-spinal fluid deteriorate rapidly with time. A routine for examining the cerebro-spinal fluid should begin with a thorough mixing of the specimen and the taking of samples for cell counts. The specimen is then centrifuged and the supernatent examined for hemolysis or discoloration. The sedimented material, if any, is then aspirated and smeared. Prior to any hematologic procedure, it should always be ascertained whether or not a bacteriologic culture is wanted from the same specimen. If so, the culture sample is taken before any other manipulations. The sediment is often examined for bacteria as well as for the differential count; a Gram stain is applied in the usual fashion.

Cell Count Procedure

1. With a WBC pipet draw filtered Unna's stain up to approximately the 0.8 mark. Expel the stain, leaving a thin film adhering to the capillary wall.

Unna's Stain: Constituents: Potassium carbonate..1 gm.
Methylene Blue.....1 gm.
Distilled water .....100 ml.

Ripen the stain in a 60°C water bath for 10 minutes. Add a crystal of thymol to prevent growth of molds.

2. Draw well-mixed spinal fluid to the "11" mark. Shake gently for two minutes.

3. Discard the first three drops from the pipet, then charge the hemocytometer chamber. Allow the hemocytometer to stand for three minutes to permit the cells to settle.

4. With a high-dry objective count and differentiate all cells in the nine square millimeter area of the counting chamber. Cells are differentiated according to their series as lymphocytes, polymorphonuclear leukocytes, or erythrocytes. Observe and report the erythrocytes as being normocellular or crenated.

5. Report the total number of cells counted and the number of each cell differentiated, utilizing a correction factor of 10/9 for the hemocytometer chamber area counted. (Observed count x 10/9).

Leukocyte counts on grossly bloody spinal fluids, are meaningless unless similar leukocyte counts can be made on whole blood. The difference between the leukocyte count of the spinal fluid and that of the blood would be that count in the cerebro-spinal fluid before the admixture of whole blood occurred. This should be compared with the red cell sediment in the spinal fluid for estimation of dilution.

Minimal degrees of xanthochromia or hemolysis can be detected by blanking with distilled water the photometer used for hemoglobin determinations and then comparing the absorption by the spinal fluid. This is a qualitative and not a quantitative procedure.

USE OF THE MICROSCOPE

The modern microscope is a precision optical instrument which permits us to make visual observations of very minute objects. Like all fine instruments, it requires care in its use and it also requires knowledge of the instrument on the part of the user. The following paragraphs are designed to acquaint you with the components of the microscope and the proper method of establishing its illumination to obtain maximum resolution from the instrument. Fig. A. shows a microscope and its lamp with a microscope board to hold them in place and to maintain proper alignment.

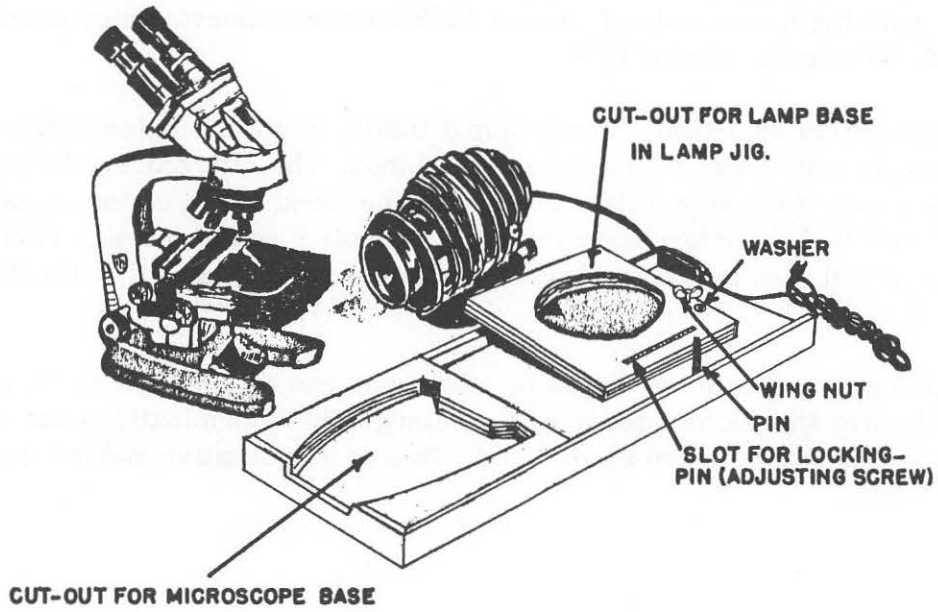


DIAGRAM OF MICROSCOPE BOARD

Figure A.

The relationship of the microscope lamp to the microscope is critical because it governs the intensity of the illumination which is available as well as the quality of the final image which the eye sees. Since microscopes are seldom in constant use, it is necessary to maintain the relative position of lamp to microscope by means of such a mount. This avoids the necessity of reestablishing the lamp-microscope relationship each time it is used. The microscope board may be fabricated locally. It is seen to consist of three essential parts: (1) the base which is a solid board with a guide strip on either side with an adjusting screw which performs as illustrated, (2) a jig for the microscope base which is permanently fixed at one end of the board, and (3) a jig for the lamp at the other end of the board. The lamp jig has a slot which permits the jig to be moved closer to or farther away from the microscope. Once the proper position of the lamp has been established, the wing nut is tightened and the lamp jig will maintain this position.

The microscope user should always sit fully erect. Slouching or bending produces unnecessary fatigue and back strain. Elevating blocks are placed under the microscope board to raise the level of the eye-pieces to the user's height.

The underside of the mounting board has holes at each of its corners. These are 9/16" deep and 2-13/16" in diameter. Fig. B. shows the underside of the microscope board and the simple blocks which can be made in assorted lengths (4", 3", 2", 1") to be nested together to provide the necessary height adjustment.

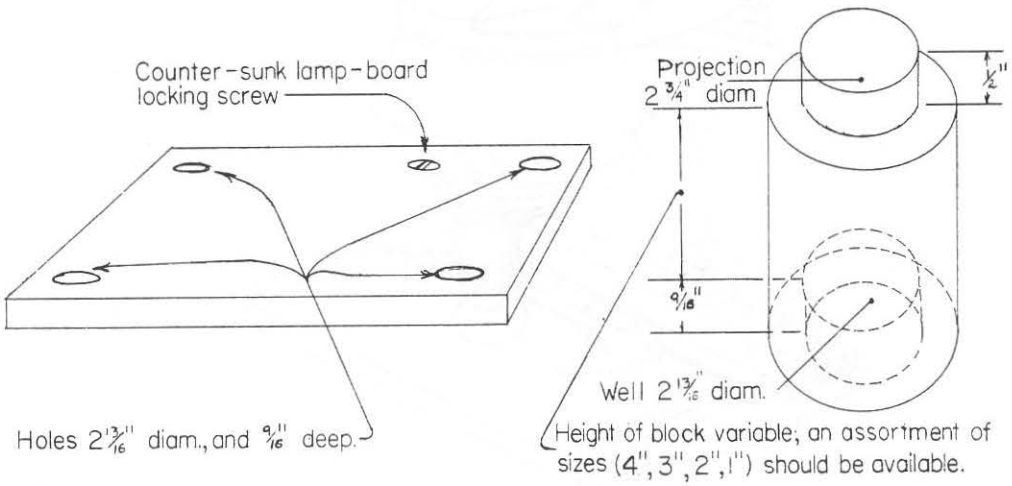


Figure B.

The dimensions listed are illustrative; other sizes can be utilized as well. It is important to have the center line of both the lamp and the microscope coinside when their jigs are positioned, or else the light beam will be out of alignment in the horizontal axis.

The proper name for the system of illumination which will be described is "Köhler illumination." Preparatory to establishing this illumination system review the components of the microscope illustrated in Figs. C and D. Fig. C shows the upper portion of the microscope. Take a hematologic slide and place it on the microscope stage. Swing the 10X objective into place. Adjust the microscope mirror so that illumination is obtained. Rack the condenser to its uppermost position. Using the coarse focus adjustment of the microscope, obtain an image of the specimen on the slide. Next, adjust the ocular focusing control (Fig. C) to zero. Adjust the interpupillary distance control (Fig. C) so that the lateral spread of the two eye pieces is comfortable to the eye and but one image is seen. While doing this you will note that the image of each eye piece fuses to produce, in our mind's eye, a single circular image. Note the scale setting which is on the front of the microscope between the eye tubes for future reference.

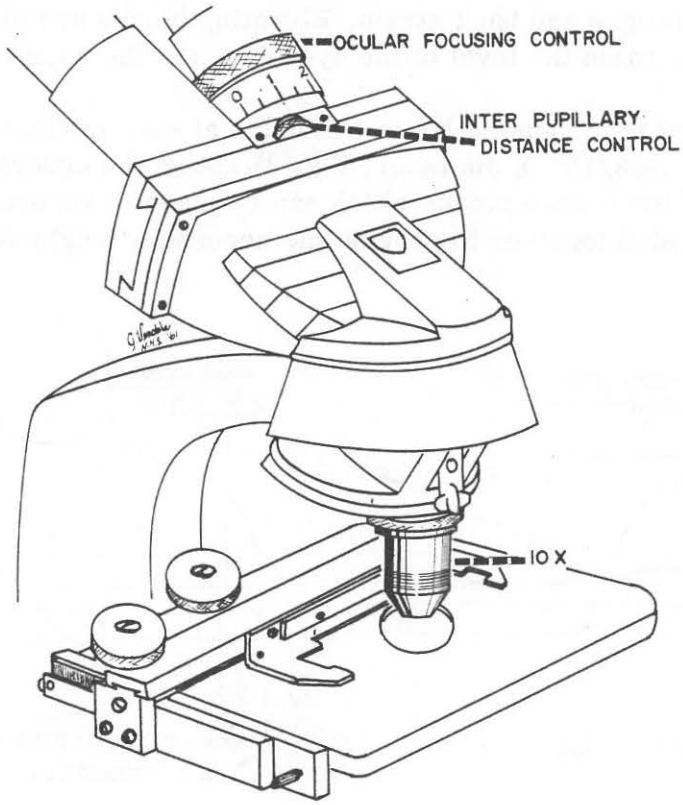


Figure C.

With the objective fine focus adjustment (Fig. D) obtain a critical focus of the object on the slide using only the eye piece which does not have the ocular focusing control. For convenience, slip a piece of opaque paper over the eyepiece of the tube with the ocular focusing control. Obtain a critical focus. Now switch the

card to the non-focusing control eyepiece so that it is obstructed. Do not further touch the objective fine focus adjustment. Using the knurled ring of the ocular focusing control, bring the image into fine focus for your eye. The microscope is now adjusted to accommodate for small differences in refraction of your two eyes. Note the numerical setting on the ocular focusing control ring for future reference.

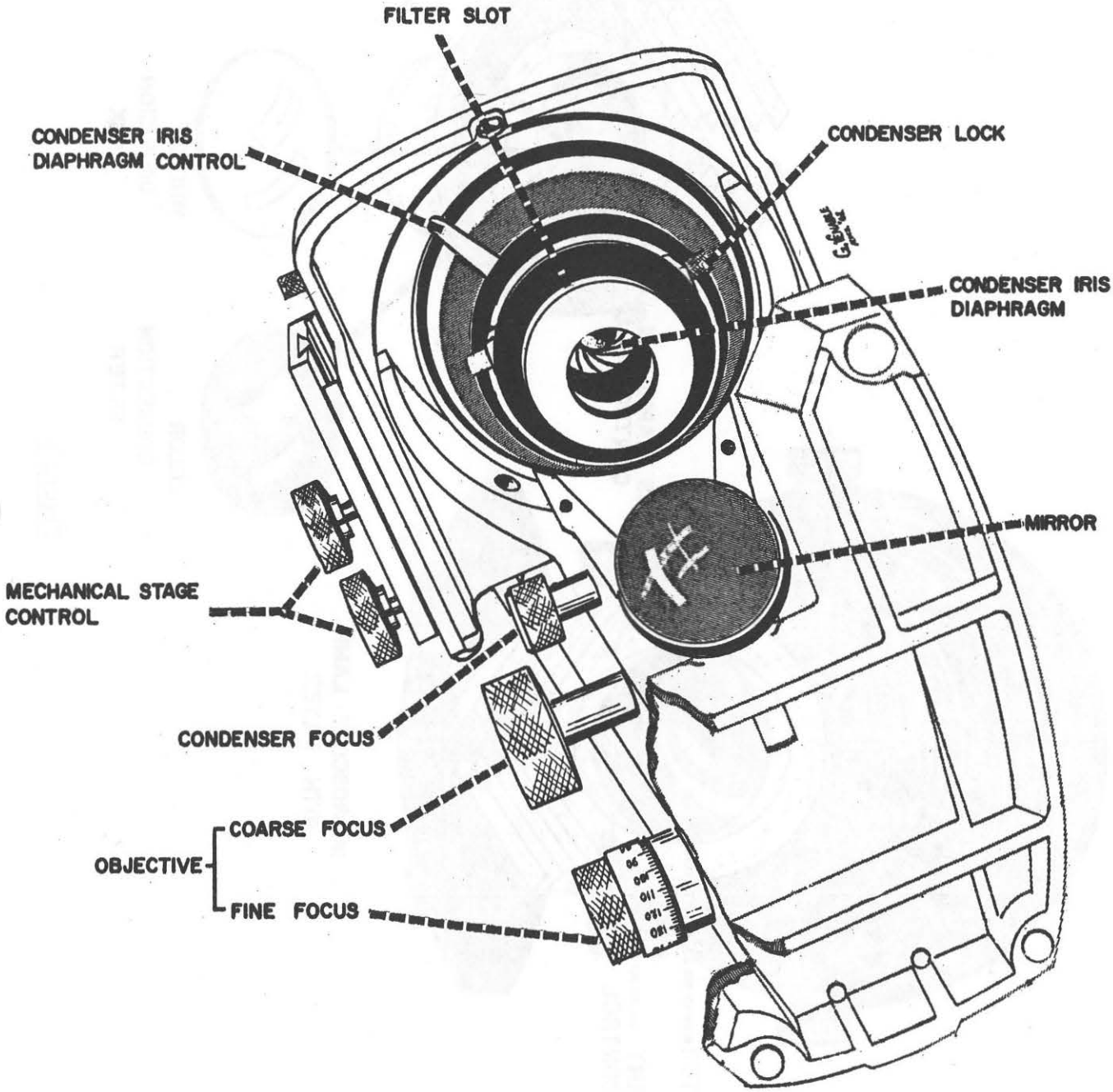


Figure D.

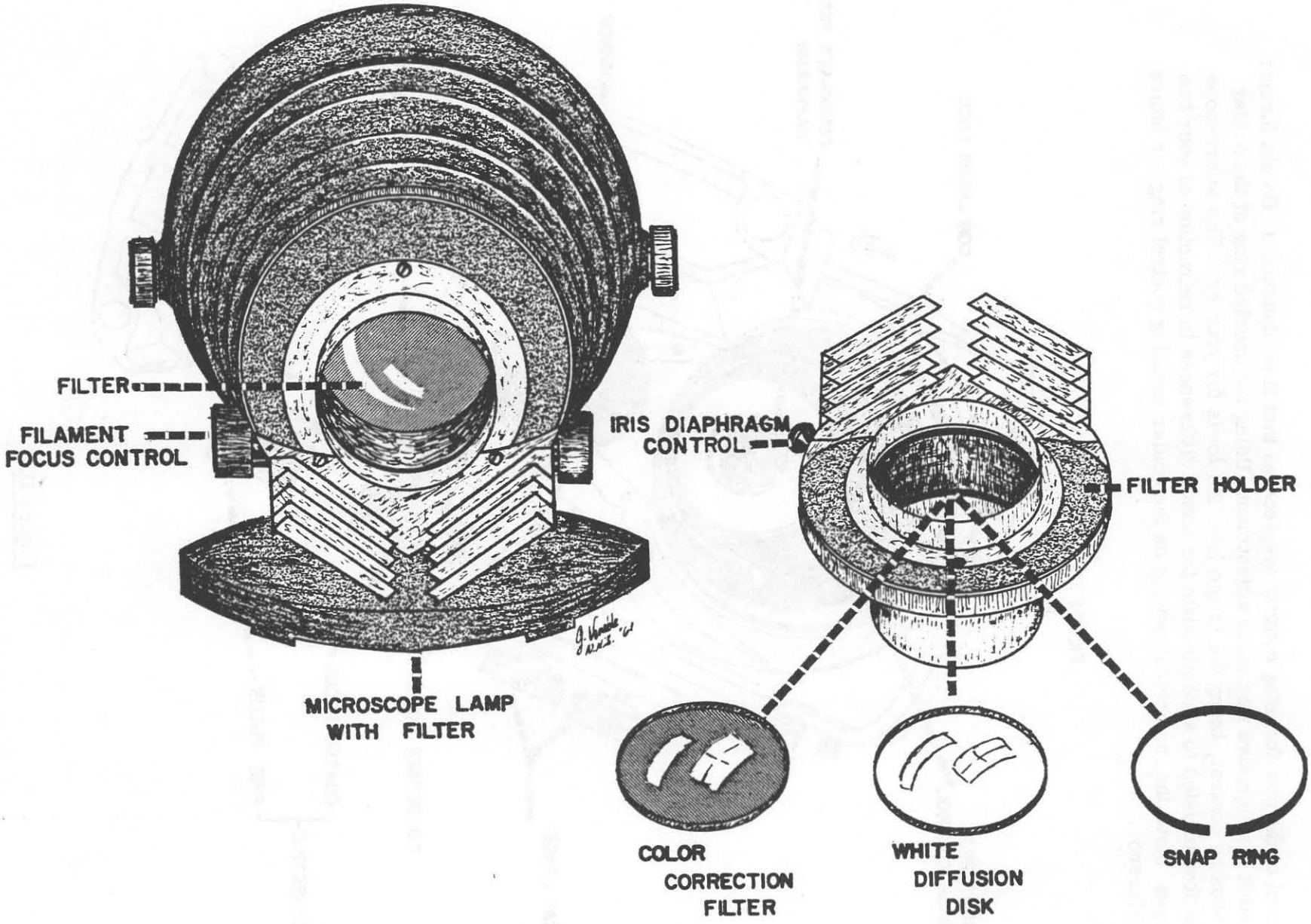

FILTER
FILAMENT FOCUS CONTROL
MICROSCOPE LAMP WITH FILTER
IRIS DIAPHRAGM CONTROL
FILTER HOLDER
COLOR CORRECTION FILTER
WHITE DIFFUSION DISK
SNAP RING

Figure E.

If you wear eyeglasses, these may be removed during microscopy. A person having astigmatism necessitating constant use of his glasses should obtain a special set of oculars designed for use with spectacles.

Next we will establish the illumination for the microscope. Once again, note the components labeled in the figures; these will be referred to in the description which follows.

Place a piece of filter paper over the mirror of the microscope. Be sure that the flat side of the mirror is facing forward. (The curved side of the mirror is never to be used with an artificial light source). Remove the filter holder from the lamp. Close the iris diaphragm of the lamp as far as it will go (shown in Fig. F).

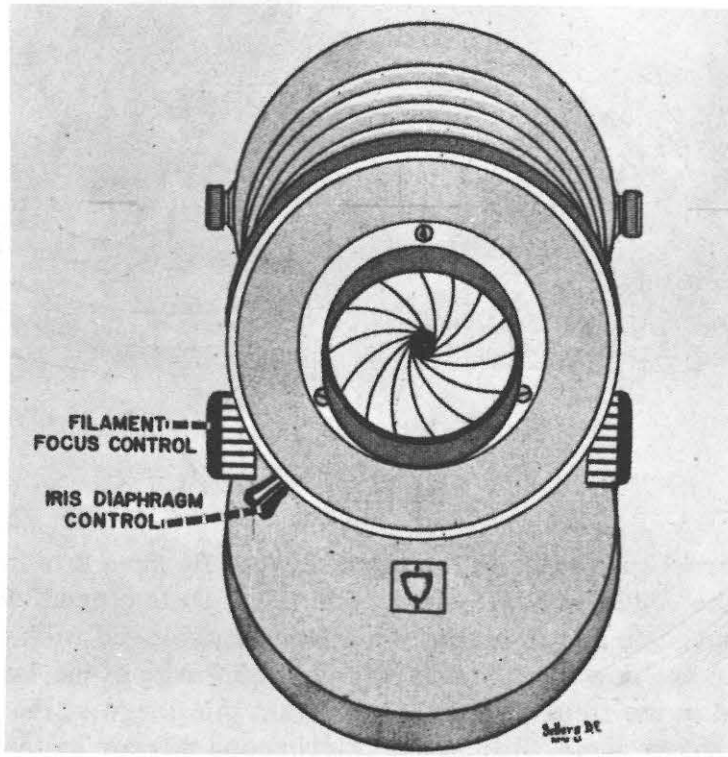


Figure F.

Do not force this or any other control. Align the lamp so that it is directly in front of the microscope and is at a distance of approximately 8 to 12 inches from the microscope. By means of the knurled knob at the back of the lamp base (seen in Fig. A and G) adjust the vertical position of the lamp so that the light falls on the center of the microscope mirror. With the filament focus control, rack the lamp tube back and forth until the filaments of the lamp bulb are sharply focused on the mirror as shown in Fig. G. Adjust the horizontal and vertical directions of the lamp so that the image of the filaments is centered on the mirror.

(Focusing the lamp filaments on the mirror is a convenient method of establishing the illumination. However, an even more accurate method is to take a second flat mirror and position it adjacent to the microscope mirror so that one can see the underside of the condenser iris diaphragm (as seen in (Fig. D). The condenser iris diaphragm is closed so that its opening is but a pin-point. Adjust the lamp filament focus controls so that the image of the lamp filaments is in focus and centered, on the underside of the condenser iris diaphragm.)

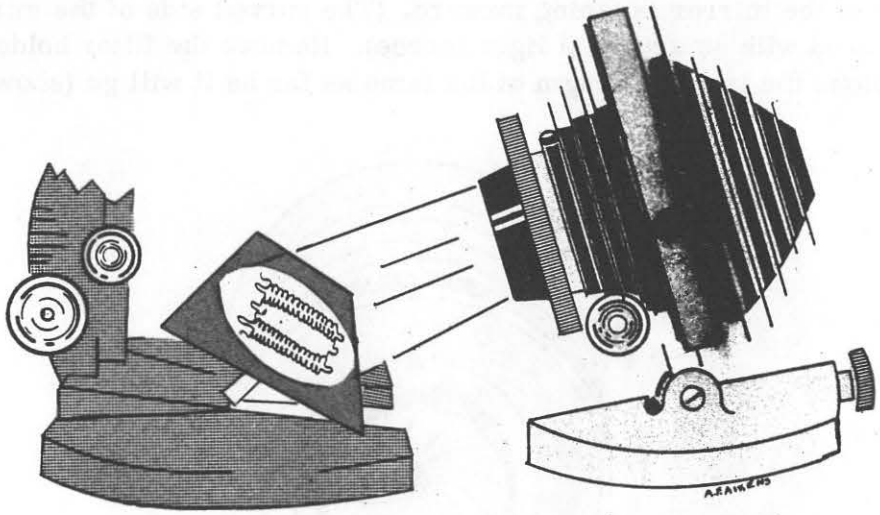


Figure G.

Check the microscope image of the slide object. Be sure this is still in focus. Now be sure that the condenser iris diaphragm (Fig. D) is closed. With both the condenser iris diaphragm and the lamp iris diaphragm closed rack the microscope condenser up and down until a sharply defined image of the lamp iris diaphragm is obtained in the field of view. Once again the image of the object on the slide should be in sharp focus. Adjust the microscope mirror so that the image of the lamp iris diaphragm is centered in the middle of the field of vision. Open the iris diaphragm of the lamp until the full field of vision seen through the microscope eyepieces is fully illuminated. When the lamp iris diaphragm image is centered and sharply defined in focus, no further adjustment to the lamp or microscope condenser is required. (Some microscopes may require an additional auxiliary lens, which is of the swing-out variety, beneath the condenser before full field illumination is obtained. This depends on the condenser attached to the microscope. In such instances the auxiliary lens is kept out of the optical pathway until the lamp adjustments and condenser adjustments are completed.)

Replace the filter holder on the lamp, being careful not to disturb the position or adjustment of the lamp. You will note that the filter holder has a blue-tinted color correction filter, a white diffusion disc, and a snap-ring to hold the tube components, color filter and diffusion disc, in place. There is room for additional filters in the external rack of the filter holder.

Once the illuminating lamp and the microscope condenser have been focused the only other adjustment which must be made is that for the condenser iris diaphragm. This iris diaphragm is NOT intended to control the level of illumination seen in the microscope. Instead it is intended to serve as a part of the optical system, which is responsible for resolution of the image. There is only one proper iris diaphragm control setting for each of the objectives on the microscope. In order to adjust properly the iris diaphragm, carry out the following steps. (See Figs. D and H.)

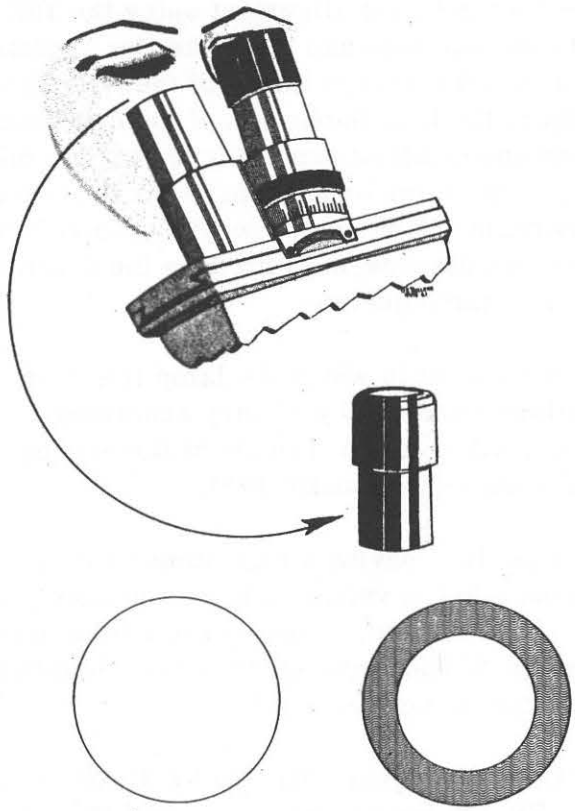


Figure H.

Open the condenser iris diaphragm fully. Remove one of the oculars (eye pieces of the microscope), as shown in Fig. H. Look down the eye tube and you

will see that the back lens of the objective is fully filled with light. Now close the iris diaphragm of the condenser to its fullest closed position. You will now see that there is but a pinpint of light visible at the back lens of the objective. Once again fully open the iris diaphragm of the condenser. Now slowly close it until you see that the diameter of the back lens' illumination is being slowly reduced. Continue to close the iris until approximately two-thirds to three-quarters of the diameter of the back lens is now fully illuminated. This leaves the outermost 1/4 to 1/3 of the back lens of the objective non-illuminated as shown in Fig. H. Replace the ocular (eye piece). Your microscope is now properly adjusted for Köhler illumination which will produce the finest resolution your microscope is capable of offering.

When your microscope has a revolving nose piece and you are using more than one objective, you will find that it becomes necessary to readjust slightly the entire series of adjustments which we have previously described. For this reason it is advisable to make your first alignment using the 10X objective. Then go to your oil immersion objective and make the fine adjustments necessary to produce a sharply focused centered image of the iris diaphragm of the lamp. Also, properly readjust the iris diaphragm of the condenser. With the lamp focus, and mirror condenser adjustments aligned for the oil immersion lens, we do not further adjust the lamp or condenser for the objectives except for the condenser iris diaphragm. Each objective will require a slightly different setting of the condenser iris diaphragm to produce the desired reduction in illumination of the back lens of the objective.

In a similar manner, the extent to which the lamp iris diaphragm must be opened to produce a fully illuminated field will vary according to the objective being used. For convenience, allow this to remain at the setting required for the lowest power objective being used (usually 10X).

Never attempt to use objectives having a high numerical aperature (N.A.) value with a condenser having a lower value. Ask your pathologist to check your microscope and its optics in this regard. However, this loss in resolution is relatively slight in comparison to that obtained when the illumination is improperly aligned for the oil immersion objective.

THE INTENSITY OF ILLUMINATION SHOULD BE CONTROLLED WITH NEUTRAL DENSITY FILTERS. Do not attempt to control the intensity of light as your eyes see it by opening or closing the condenser iris. This is incorrect. Likewise, you cannot control the intensity of illumination with the lamp iris. The lamp iris is used only to cut out extraneous light once it has been opened sufficiently to produce a full field of illumination. The intensity of illumination may be controlled by regulating the lamp voltage, in which case suitable color correction filters to produce a white or blue-white light are required. If the

voltage cannot be controlled we must use neutral density filters to reduce the amount of light which we receive. Within limits, the intensity of illumination may also be controlled by placing the illuminating lamp closer to, or further from, the microscope. Once one has properly established the relationship of microscope lamp to the microscope and adjusted the lamp and condenser focus, then these relationships can be maintained by locking the wing-nut on the lamp board. From that time on the lamp and microscope may be removed from the board for storage, then brought back into use without further adjustments by simply placing them in their respective jigs, provided none of the settings previously described has been disturbed.

GLOSSARY OF TERMS

Agranulocytosis: complete or nearly complete absence of the granular leukocytes from the blood and bone marrow.
Anemia: reduction below normal in the number of erythrocytes and/or quantity of hemoglobin.
Anisocytosis: variation in size of the erythrocytes.
Anoxemia: lack of normal proportion of oxygen in the blood.
Aplasia: incomplete or defective development; cessation of blood cell manufacture.
Azurophil granules: rounded, discrete, reddish-purple granules, larger than the granules of neutrophils. Common in lymphocytes. They are very numerous and smaller in the cytoplasm of monocytes.

Basophilia: (1) Staining readily with basic dye (blue with Romanowsky type stains). (2) an increase in basophils.
Buffy coat: the layer of leukocytes that collects immediately above the erythrocytes in sedimented or centrifuged whole blood.

Cabot's ring bodies: thread-like rings, convolutions and figures of eight seen in erythrocytes in severe anemias.
Chemotaxis: the phenomenon of movement of leukocytes to site of injury caused by a chemical influence.
Chromatin: the more stainable portion of the cell nucleus.
Congenital: born with a person; existing at or before birth.
Crenation: the scalloped or notched appearance of the periphery of erythrocytes found when the cells are suspended in a hypertonic solution. Also found in smears - caused by dirty glassware, slow drying, and poor smearing technic.

Disintegrated cell: ruptured leukocyte.
Dohle bodies: pale blue bodies found in the cytoplasm of the neutrophil indicating a toxic condition.
Drepanocyte: see Sickle cell.
Dyscrasia: abnormality.

Erythremia: a disease marked by persistent increased red count and increased blood volume; also polycythemia vera.
Erythrocyte: red blood cell.
Erythropoiesis: the production of erythrocytes.
Etiology: the theory of the causation of a disease.

Fenestration: perforation.

Granulopoiesis: the production of granulocytes.

Hematin: a substance composed of iron and protoporphyrin which with globin forms hemoglobin.
Hematopoietic: blood forming.
Hemoglobinuria: hemoglobin free in the urine.
Hemolysis: the dissolution of the erythrocytes.
Heterozygous: derived from germ cells unlike in respect to one or more factors.
Homozygous: derived from like germ cells, or zygotes.
Howell-Jolly bodies: nuclear remnants as coarse, round basophilic particles.
Hyperplasia: an increase in cell formation.
Hypochromia: increased central pallor due to lack of hemoglobin.
Hypoplasia: a decrease in cell formation.

Idiopathic: of unknown cause.
Intrinsic factor: a specific factor with enzyme-like properties present in normal gastric secretions.

Leptocyte: hemoglobin eccentrically arranged in the cell resembling a target.
Leukemoid crisis or reaction: a temporary appearance of immature leukocytes in the blood stream with a marked increase in the total white count. In the laboratory sometimes temporarily indistinguishable from leukemia.
Leukocyte: white blood cell.
Leukocytosis: an increase in leukocytes in the blood.
Leukopenia: reduction in the number of leukocytes in the blood.
Leukopoiesis: leukocyte formation.

Macrocyte: cell larger than normal.
Mast cell: a tissue basophil.
Maturation factor: a substance which will cause cells to ripen and come to maturity.
Methemoglobin: a spectroscopically detected compound of hemoglobin found in nitrobenzol and other poisonings. The blood is a chocolate brown color to the eye.
Microcyte: a cell smaller than normal.
Micron: 1/1000 of a millimeter; the common unit of measurement of microscopic objects.
Mitosis: a series of changes through which the nucleus passes in indirect cell division. A tissue showing many cells in mitosis indicates rapid growth of that tissue.

Necrosis: the death of a circumscribed portion of tissue. Simple necrosis is degeneration of the cytoplasm and nucleus without change in the gross appearance of the tissue.
Nucleolus: a homogeneous blue-staining area within the nucleus of a cell.
Neutropenia: decrease in neutrophils.
Neutrophilia: increase in neutrophils.

Occult blood: blood that cannot be detected except by chemical test.
Ovalocyte: an elliptical erythrocyte.

Pathologic increase (or decrease): due to abnormal function or disease, as contrasted to physiological (due to normal body function).
Petechiae: small spots on the skin formed by subcutaneous effusion of blood, also purpura and ecchymoses.
Phagocytosis: the destruction of organisms and extraneous matter by a process of envelopment and absorption.
Plasma: the fluid portion of the blood composed of serum and fibrinogen; obtained when an anticoagulant is used.
Poikilocytosis: variations in shape of erythrocytes.
Polycythemia: an increase in the total number of erythrocytes. See erythremia.
Punctuate basophilia: small basophilic particles in the erythrocytes that stain blue with the basic dye of Wright's stain; also basophilic stippling.
Purpura: see petechiae.
Pyknosis: a condensation and reduction in size of the cell and its nucleus.

Reticuloendothelial cell: a cell with reticular and endothelial attributes; concerned in blood cell formation, bile formation, and phagocytic destruction of blood cells.

Serum: the fluid portion of clotted blood.
Sickle cell: a sickle or crescent-shaped erythrocyte.
Spherocyte (microspherocyte): small, round, deep staining red cells without central pallor.
Supravital stain: the staining of living tissue removed from the body.

Target cell: see leptocyte.
Thrombocyte: platelet.
Thrombocytopenia: a decrease in thrombocytes; also thrombopenia.
Thrombocytosis: an increase in thrombocytes.
Thrombosis: formation of a thrombus or blood clot.

Vacuole: a space or cavity formed in the protoplasm of a cell.

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